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Characterization Of Antimicrobial-Resistant staphylococcus Spp. In Food Animals And Retail Meat

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CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT *STAPHYLOCOCCUS* SPP. IN FOOD ANIMALS AND RETAIL MEAT

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DEDICATION

This work is dedicated to

My parents and almighty for guiding me and showing me the right path

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LIST OF ABBREVIATIONS

agr - accessory gene regulator

ATCC - American Type Culture Collection

BHI - Brain Heart Infusion

CA-MRSA - community associated methicillin resistant *Staphylococcus aureus*

ccr - cassette chromosome recombinase

CDC - Centers for Disease Control and Prevention

CFU - colony forming units

CLSI - Clinical and Laboratory Standards Institute

CoNS - coagulase-negative staphylococci

DNA - deoxyribonucleic acid

dru - mec-associated direct repeat unit

EDTA - ethylenediaminetetraacetic acid

MDR - multidrug-resistant

MIC - minimal inhibitory concentration

MLST - Multi-Locus Sequence Typing

MRCoNS - methicillin-resistant coagulase negative Staphylococci

MRSA - methicillin-resistant *Staphylococcus aureus*

MSSA - methicillin-susceptible *Staphylococcus aureus*

MHA - Mueller Hilton Agar

PBP - penicillin binding protein

PCR - polymerase chain reaction

PFGE - pulsed field gel electrophoresis

PVL - Panton-Valentine leukocidin

Q/D - quinupristin-dalfopristin

SCC*mec* - Staphylococcal Cassette Chromosome *mec*

spa - staphylococcal protein A spa gene

ST - sequence type

US - United States of America

VRSA - vancomycin-resistant *Staphylococcus aureus*

BACKGROUND AND SIGNIFICANCE

General Overview

Antimicrobial resistance is an inevitable consequence of evolutionary adaptation of microbes and has emerged as epidemic crisis in clinical and veterinary medicine worldwide. Human use and misuse of antimicrobial drugs have driven the increasingly rapid and prevalent emergence of resistance in both pathogenic and commensal organisms (Silbergeld *et al.*, 2008). Also, Industrial food animal production and utilization of antimicrobials lead to an increase of antimicrobial resistance in animals. Antimicrobials are used for therapy as well as in animal feed to increase the growth rate, improve feed conversion, and reduce morbidity and mortality (Boerlin, 2010). A wide range of antimicrobial drugs representing all the major classes of clinically important antimicrobials, from penicillin to third generation cephalosporin, are permitted for use in animal production in the US (Sapkota *et al.*, 2007).

Food production environment is an important reservoir of multidrug-resistant commensals and pathogens. Given the potentially endless cycle of spread of these antimicrobial resistant bacteria in animal and humans, transmission may occur by direct contact or indirectly, through food, water and animal waste application to farm fields (Marshall and Levy, 2011). Although a far reaching and complex route of transmission, consumers may be exposed to resistant bacteria via contact or consumption of animal products. Investigation of molecular epidemiology, transmission and emergence of these pathogens in food animals and retail meat is the foundation of developing, implementing, and evaluating an effective control programs to prevent the occurrence of human infections due to human consumption of food carrying antimicrobial resistant bacteria. One of the strategies to encounter these pitfalls is to explore the

power of natural products such as phyto-chemicals as antimicrobial and antimicrobial adjuvant to control or prevent the infections by multidrug resistant pathogens (Aiyegoro and Okoh, 2009).

Genus *Staphylococcus*

Microbiology

Members of the genus *Staphylococcus* are nonsporulating, nonmotile, halotolerant, Gram-positive cocci that have an average diameter of 1µm and microscopically appear as grapelike clusters. They are low GC bacteria (33-40mol %) which are usually facultative anaerobes and catalase positive. *Staphylococcus* can grow in the presence of 10% NaCl and between 18-40°C (Götz *et al.*, 2006; Schleifer *et al.*, 1981). The genus includes 39 validly described species according to list of prokaryotic names with standing in nomenclature (www.bacterio.net). Eleven of these comprised of two or more subspecies resulting in more than 50 systematic entities (Blaiotta *et al.*, 2010; Euzéby, 1997).

On the basis of coagulase production, they are classified into two groups: coagulase-positive staphylococci such as *S. aureus, S. pseudintermedius*, S. *intermedius, S. schleiferi* subsp. *coagulans, S. hyicus, S. lutrae,* and *S.delphini,* and coagulase-negative staphylococi (CoNS) such as *S. epidermidis, S. sciuri, S. lentus, S. saprophyticus* and many more (Kloos, 1884). On blood agar, staphylococci form 1-2 mm round and smooth colonies that are often pigmented and may be surrounded by β-hemolysis zone (Chanda *et al.*, 2010). The glycine interpeptide bridge in peptidoglycan of cell wall make them susceptible to lysis by lysostaphin, but resistant to lysis by lysozyme (Kloos, 1884).

Staphylococci are ubiquitous in nature. They are one of the most important bacteria among the natural skin flora of mammals and birds. Colonizer of the skin or mucosal membranes

of the respiratory, upper alimentary, and urogenital tracts, they can easily spread between animals and humans through contact or vectors. The different hosts of staphylococcal species include mammals, birds, primates, humans, food, and domestic animals (Aarestrup, 2006). They were primarily known as nosocomial pathogens, however, the frequency of community acquired infections has increased in the past few years (Martins and Cunha, 2007).

Clinical Significance

Staphylococcus aureus, one of the most pathogenic member of coagulase positive group of Staphylococci, is responsible for a wide variety of human ailments including skin, soft tissue and bone infections, pneumonia, and bacteremia etc (Lipsky *et al.*, 2007). They are capable of producing several virulence factors such as Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), enterotoxins serotypes A through Q, cytolytic and exfoliative toxins (McCormick *et al.*, 2001). These strains can survive and multiply in food, and are well-known for outbreaks of foodborne illness (Le Loir *et al.*, 2003). Other pathogenic members of the group include *S. intermedius* and *S. hyicus*, which are capable of causing severe infections in animals. *S. intermedius* has been implicated in a variety of infections in dogs, whereas *S. hyicus* is of concern in poultry and pigs (Phillips and Kloos, 1981).

The majority of species in genus *Staphylococcus* belongs to CoNS, which is the most diverse group of commensals inhabiting the skin and mucous membrane of animals and humans. CoNS were considered unimportant with regard to their role causative agents of infections until 1980s. Since then, CoNS have been known as important opportunistic human and animal pathogens. They are number one bacterial agent in monomicrobial nosocomial bloodstream infections in the US, followed by *Staphylococcus aureus* and enterococci (Zhang *et al.*, 2009). *S.*

epidermidis is the most frequent source of CoNS infection such as intravascular catheter-related infections, nosocomial bacteremia, endocarditis, urinary tract and surgical wounds infections, central nervous system shunt infections, ophthalmologic infections, peritoneal dialysis-related infections and infections of prosthetic joints (Widerstrom *et al.*, 2012). In animals, CoNS are capable of causing several animal diseases such as suppurative disease, mastitis, arthritis and urinary tract infection (Lee, 2003). Other CoNS species of clinical significance includes *S. hemolyticus*, *S. saprophyticus*, *S. lugdunensis* and members of *S. scuiri* group (Piette and Verschraegen, 2009). *S. scuiri* species group is composed of species *S. scuiri, S. lentus, S. vitulinus* and *S. fleuretti.* Dominant colonizer of rodents, squirrels and other animals, they have been associated with severe infections in humans such as endocarditis, septic shock, urinary and wound infection (Stepanovic *et al.*, 2003). Likewise, *S. saprophyticus* is well-known to cause urinary tract infections in young women and eating contaminated food may lead to colonization and infection (Widerstrom *et al.*, 2012). Therefore, clinical significance and reservoir of staphylococci may vary with species. Overall, infections caused by genus *staphylococcus* which is widely distributed in nature are of great importance for animal and public health (Martins and Cunha, 2007).

Isolation Procedures

Staphylococcus aureus is a well-known food borne pathogen capable of causing food poisoning. Its presence in food, therefore, is a food safety concern. According to Bacteriological Analytical Manual (BAM) protocol, the food slurry should be diluted in Tryptic Soy Broth containing 10% NaCl and then examined for the presence of *S. aureus* on selective agar such as Baird Parker agar/Vogel-Jhonson Agar (Holbrook *et al.*, 1969; Leininger, 1976; Food, 2004). In addition, it is highly suggested to add filter sterilized catalase or pyruvate to TSB containing 10%

NaCl for good recovery of heat stressed *S. aureus* cells from food (Brewer *et al.*, 1977). Direct plating is preferred in laboratories for enumeration of staphylococci than Most Probable Number (MPN) procedures. Baird Parker Agar (BPA) is majorly used, however, Tellurite polymyxin egg yolk agar, Sheep Blood agar (5% defibrinated sheep/rabbit/bovine blood), Columbia CAN agar, phenylethyl alcohol agar, and Mannitol salt agar (MSA) are some other selective medium which are being successfully applied for isolation of Staphylococci (Götz *et al.*, 2006).

Procedures used in isolation of staphylococci from clinical samples involved direct plating on blood agar containing 5% sterile defibrinated sheep (preferred), rabbit or bovine blood. The inoculated plates are incubated at 34-37°C for at least 18-24 h under aerobic conditions in order to isolate typical colonies of staphylococci. Recently, CHROMAgar *Staph aureus* (CSA, BD-BBL) and CHROMAgar MRSA (BD-BBL), chrom ID (bioMérieux), MRSASelect (Bio-Rad) and Brilliance MRSA (Oxoid), all of which are based on the use of specific chromogenic substrates, were developed for the isolation of *S. aureus* and MRSA from clinical samples (Bautista-Trujillo *et al.*, 2013). The swab technique is satisfactory for isolation of staphylococci from humans, animals, and environmental samples. The primary isolation plate may be made up of blood agar, trypticase soy agar, brain heart infusion agar or other non selective media (Kloos, 1884).

Genus and Species level Identification

Staphylococcus genus can be identified on the basis of typical characteristics of Staphylococci. Gram and catalase positive cocci, they can easily grow in a media containing 7.5% NaCl when incubated for 24 hours at 35°C (Kloos, 1884). Members of *Micrococcus* genera may be differentiated from Staphylococci because of their ability to produce acid from mannitol (Götz *et al.*, 2006). Furthermore, molecular biological tests are available for confirmation. PCR

based amplification of 16S rRNA gene for *Staphylococcus* spp. is highly recommended and a widely used method for confirmation of suspected Staphylococci strains (Chakravorty *et al.*, 2007).

Accurate identification of *Staphylococcus* spp. is of prime importance in both human and veterinary laboratories. Numerous phenotypic and genotypic methods are used to identify species of Staphylococci. However, phenotypic methods are dependent on expression of morphological or metabolic activity which are often difficult to identify (Schleifer *et al.*, 1981). Several biochemical tests based kits and automated identification systems for *Staphylococcus* spp. are available. The test kits consist of strips or trays with microcupules or wells containing dehydrated substrates, biochemicals, and/or nutrient media. The strips/trays are incubated for 5-24 hr after inoculation followed by interpretation of results. One of the examples of these systems is API-Staph-IDENT (Kloos and Wolfshohl, 1982). The automated systems are utilized by several large laboratories. However, in settings where automated systems are not available, it is a common practice to consider existing information about the isolates such as source and sampling site. Initial isolation on selective medium such as MSA and/or BPA is followed by gram staining and coagulase test. *S. aureus* produce yellow halos around the colonies on MSA due to production of acid from mannitol (Winn and Koneman, 2006). Other Staphylococci grow well on MSA without fermentation and competing bacteria who cannot survive in 7.5% NaCl are automatically eliminated at this step. Specifically, for rapid confirmation of *S. aureus* several chromogenic agar and latex agglutination tests are available in the market (Smole *et al.*, 1998).

Since, phenotypic discrimination cannot reliably identify species because of variable expression of phenotypic traits (Irlinger, 2008). Currently, several laboratories employ molecular tools such as PCR and DNA sequencing for genus and species identification. Numerous targets

have been identified including 16S rRNA, the 16S-23SrRNA intergenic spacer region, *hsp*60 (heat shock protein 60), *sod*A (superoxide dismutase A), *rpo*B (the RNA polymerase β subunit) and *fem*A (factor responsible for methicillin resistance) (Zadoks and Watts, 2009). DNA sequences of these genes are compared with sequences stored in large publicly available databases to determine species and subspecies.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometery (MALDi-TOF MS) is a recent method for species identification showing a great promise for identification and typing of staphylococci (Widerstrom *et al.*, 2012). Recent publications have demonstrated versatility and reliability of this method for identification of staphylococcal species and subspecies (Dubois *et al.*, 2010; Szabados *et al.*, 2010).

Subtyping

Molecular typing techniques have been developed to gather the thorough knowledge of both dissemination and the epidemiology of MRSA and other staphylococci. Many different phenotypic and genotyping methods are currently in use for subtyping. Phenotypic methods such as biochemical activity, antimicrobial susceptibility, serological and phage typing lack discriminatory power on closely related strains. Genotypic methods offer higher resolution and have become method of choice for strain typing. Pulsed Field Gel Electrophoresis (PFGE) is the current gold standard in microbial subtyping and offers considerable discriminatory power, with high degree of typeability (Stefani *et al.*, 2012). Other techniques that are used include PCR-based methods, such as Staphyloccocal cassette chromosome *mec* (SCC*mec*), accessory gene regulator (*agr*), *mec*-associated direct repeat unit (*dru*) typing, and more recently DNA-sequencing approaches like Multi-Locus Sequence Typing (MLST) and staphylococcal protein A

(*spa*) gene typing (Cookson *et al.*, 2007). Few commonly used subtyping methods are described in detail below:

Pulsed Field Gel Electrophoresis (PFGE)

This method has been demonstrated as one of the most discriminative typing methods for studying outbreak and hospital to hospital transmission of MRSA. Purified chromosomal DNA carefully extracted low melting point agarose plugs is digested by restriction enzyme *Sma*I followed by agarose gel electrophoresis, staining and visualization. Adequate separation of both small and large DNA fragments is accomplished by pulsed-field gel electrophoresis – electrophoresis employing alternately pulsed, perpendicularly oriented electrical fields because conventional gel electrophoresis does not adequately separate the largest similarly-sized DNA fragments (Schwartz and Cantor, 1984). The banding patterns can be analyzed visually or by software's to identify similarities and differences. The most common method to construct a similarity tree diagram is "unweighed pair group method using arithmetic averages" (UPGMA) (Tenover *et al.*, 1995). Database of PFGE patterns for *S. aureus* has been generated and clinical isolates have been given numerical designations by CDC such as USA300 or USA100. Inter-laboratory differences in the result, expensive equipment, and laborious protocols are the major disadvantages of this technique (Struelens *et al.*, 2009). However, large databases of PFGE patterns of MRSA still mark it is gold standard for molecular epidemiological studies.

Multilocus Sequence Typing (MLST)

MLST is based on identification of highly conserved housekeeping genes using PCR and DNA sequencing. *S. aureus* MLST requires characterization of seven housekeeping genes (Table 1). DNA sequencing data for each locus is uploaded to MLST database (http://saureus.mlst.net) to assign allelic profiles and STs. Numerical designations are given to variation in loci. Finally,

combinations of allelic numbers are used to assign ST (Robinson and Enright, 2004). For example, isolates exhibiting MLST profile 3-35-19-2-20-26-39 are termed as ST398. Evolutionary descent and clonal complexes are identified using eBURST algorithm (http://eburst.mlst.net).

Table 1. Seven housekeeping genes and their sequence length for MLST

Housekeeping Gene	Sequence length (bp)
Carbamate kinase (*arcC*)	456
Shikimate dehydrogenase (*aroE*)	456
Glycerol kinase (*glpF*)	465
Guanylate kinase (*gmk*)	429
Phosphate acetyltransferase (*pta*)	474
Triosephosphate isomerase (*tpi*)	402
Acetyl coenzyme A acetyltransferase (*yqiL*)	516

***Spa* Typing**

Molecular typing of MRSA strains using this single-locus sequence typing method involves DNA sequence analysis of the polymorphic X-region of the protein A gene (*spa*) comprising variable number of small repeats (Frenay *et al.*, 1996). Diversity of this region consisting of mainly 24-bp repeats is attributed to deletions and duplications of repeats (Kahl *et al.*, 2005). It is a very popular method due to its simplicity and discriminatory power which lies between PFGE and MLST (Malachowa *et al.*, 2005).

SCC*mec* typing

According to epidemiological studies, SCC*mec* typing is required along with MLST and *spa* typing for proper methicillin resistant staphylococci clone assignment (Turlej *et al.*, 2011). Methicillin resistance in staphylococci is conferred by presence of 2.1 kb *mec*(A) gene on mobile genetic element designated *Staphylococcus* cassette chromosome *mec* (SCC*mec*). SCC*mec* element share four common features such as presence of *mec*(A) gene in *mec* gene complex; carriage of site specific recombinases referred to as cassette chromosome recombinases (*ccr*) genes in ccr gene complex; integration site sequences (ISS) for SCC*mec*, and the existence of flanking direct repeat sequences containing ISS. The *mec* gene complex includes *mec*(A) gene, the *mec*(I) gene the *mec*(R1) gene, and associated insertion sequences, however, *ccr* gene complex included the *ccr* genes and open reading frames (ORFs). The combination of *ccr* gene complex and *mec* gene complex, essential regions of cassette, is used to designate SCC*mec* types of methicillin resistant staphylococci (Hanssen and Ericson Sollid, 2006). Specifically for MRSA, 11 SCC*mec* types are recognized till date (Table 2) (Zong *et al.*, 2011). Besides *mec* and *ccr* gene complex, various joining regions (J regions) have been identified in some cassettes which may carry additional resistance genes, and may be used for sub typing of SCC*mec* elements (Kondo *et al.*, 2007). Presence of other elements including antimicrobial resistance genes, insertion elements such as IS*431*, plasmids such as pT181, or transposons such as Tn*554* in SCC*mec* elements may vary.

Table 2. Summary of SCC*mec* types currently described in MRSA

SCC*mec* type[1]	*mec* class[2]	*ccr* type[3]
I	B	A1B1
II	A	A2B2
III	A	A3B3
IV	B	A2B2
V	C2	C1
VI	B	A4B4
VII	C1	C1
VIII	B	A4B4
IX	C2	A1B1
X	C1	A1B6
XI	E	A1B3

1 SCC*mec* types I to XI have been assigned to *S. aureus* according to http://www.sccmec.org/Pages/SCC_TypesEN.html

2 Class of *mec*: A, IS*431*-*mec*(A)-*mec*(R1)-*mec*(I); B, IS*431*-*mec*(A)-Δ*mec*(R1)-IS*1272*; C1, IS*431*-*mec*(A)-Δ*mec*(R1)-IS*431* (two IS*431* in the same direction); C2, IS*431*-*mec*(A)-Δ*mec*(R1)-IS*431* (IS*431* in opposite direction); D, IS*431*-*mec*(A)-Δ*mec*(R1); E, *blaZ*-mec_{LGA251}-$mec(R1)_{LGA251}$-$mec(I)_{LGA251}$.

3 *ccr* type: A, ccrA; B, *ccrB*; C1, *ccrC1*.

Adapted from reference (Zong *et al.*, 2011)

Several methods for SCC*mec* typing of staphylococci are available. First SCC*mec* typing method for detecting polymorphism in the *mec*(A) vicinity was based on hybridization of *mec*(A) probe and Tn*554* probe with *Cla*I digested genomic DNA (Leski *et al.*, 1998) . During the past several years, multiplex PCR based SCC*mec* typing methods have been developed. The first PCR method described by Oliveria et al. (Oliveira and de Lencastre, 2002; Milheiriço *et al.*, 2007) relied upon specific genes and motifs in the junkyard region. Due to several disadvantages associated with this approach, SCC*mec* typing methods based on *ccr* and *mec* gene complex, became popular (Zhang *et al.*, 2005; Hansen *et al.*, 2004). In the light of new guidelines for SCC*mec* element classification, the most promising system for SCC*mec* assignment was developed by Kondo et al. (Kondo *et al.*, 2007) and it is highly recommended by researchers. The methods based on realtime PCR have also been developed (Chen *et al.*, 2009). They are fast, less labor intensive and are easy to interpret. However, they require special equipment and are very expensive. Currently, there is no method available for SCC*mec* type VII and X-XI typing.

In summary, genetic subtyping methods have a greater discriminatory power than conventional methods, but the use of multiple subtyping methods may further improve discriminatory power and may therefore be appropriate for epidemiological investigations. PFGE, SCC*mec*, MLST and *spa* typing have been most widely used to characterize MRSA and other staphylococci from different sources (Martins and Cunha, 2007).

Antimicrobial Resistance in *Staphylococcus*

History

Staphylococci were the first ones to acquire penicillin resistance due to acquisition of penicillinase encoding plasmids soon after the introduction of penicillin in early 1940s. More than 80% staphylococcal isolates were resistant by the late 1960s. Therefore, penicillinase stable

antimicrobial such as methicillin was introduced in 1961. Its introduction was followed by several reports on isolation of MRSA throughout the world (Lowy, 2003). The major challenge in treatment of *staphylococcus* infections is the emergence of resistance to all beta lactam antimicrobials including methicillin (Livermore, 2000). Treatment of infections is compromised worldwide due to the emergence of staphylococci that are resistant to multiple antimicrobials. Interestingly, the pattern of resistance was the same with each wave of resistance. The resistant strains first emerged in hospital and then transmitted to the community. Recently, four waves of antimicrobial resistance in *S. aureus* have been described (Chambers and DeLeo, 2009). The introduction of penicillin and methicillin marks as an onset of first and second wave of resistance. Emergence of new strains of MRSA such as MRSA-II and III demonstrated third wave of resistance in hospital and health care facilities in late 1970s. In late 1990s, CA-MRSA emerged in community with the fourth wave of resistance. These strains carried SCC*mec* IV and virulence factor such as PVL. Finally, VRSA was identified in 2002 in hospitals. Quinupristin-dalfopristin, linezolid and daptomycin are the current antimicrobials with activity against VRSA. New agents offer a renewed opportunity to control but staphylococci are well-known as extraordinary adaptable pathogen with proven ability to develop resistance.

Antimicrobial Resistance Mechanisms

Intrinsic or acquired mechanisms are responsible for development of bacterial antimicrobial resistance. Naturally occurring genes in host's chromosome specifies intrinsic mechanism. Acquired resistance involves transfer of resistant determinants on plasmids, bacteriophages, transposons, and other mobile genetic material. Exchange of genetic material is achieved through conjugation, transformation and transduction (Alekshun and Levy, 2007). Furthermore, acquired resistance is also accomplished by mutations in genes targeted by

antimicrobial. Plasmid containing resistance genes may replicate independently in host machinery and transposon can exist on plasmid or other transposon or host chromosome. Thus resistant determinants can transfer easily utilizing these mobile genetic materials (Levy and Marshall, 2004).

Antimicrobial resistance develops predominantly due to four reasons including mutated drug target, destruction or modification of antimicrobial by enzyme produced by organism, efflux of antimicrobial from cell or altered membrane permeability (Walsh, 2003). MRSA is one of the most problematic organisms in which novel pencillin binding protein produced by *mecA* is responsible for methicillin resistance. Although, alteration of penicillin binding protein is the primary mechanism of resistance to beta lactam, MRSA strains have gained multiple mechanisms of resistance to several classes of antimicrobials including macrolides, aminoglycosides, fluroquinolones, tetracyclines, and vancomycin (Lowy, 2003).

MRSA

MRSA is a type of bacteria that is resistant to methicillin and other β-lactam antimicrobials, such as oxacillin, penicillin and ampicillin. In general, MRSA infections have been categorized into four groups based upon their sources: health care-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA), health care associated MRSA with community onset and livestock associated MRSA (LA-MRSA) (Price *et al.,* 2012). Although MRSA infections mainly occur in hospitals, human illness caused by community-associated MRSA (CA-MRSA) is increasing substantially (Kennedy *et al.*, 2008). CA-MRSA infections are more severe if the bacteria produce Panton-Valentine leukocidin (PVL), an *S. aureus*-specific exotoxin associated with severe skin infections and necrotizing pneumonia. Genetically

heterogenous, CA-MRSA includes a variety of clones such as ST 1 (USA400 clone) and ST8 (USA300 clone), which is a major CA-MRSA clone in the US (Yabe *et al.*, 2010). This well known nosocomial agent and community-associated pathogen has become the focus of concern as it has developed as LA-MRSA like Sequence Type (ST) 398 (Smith and Pearson, 2011; Vanderhaeghen *et al.*, 2010).

MRSA expresses a novel penicillin-binding protein, (PBP)-2a, which is encoded by the chromosomal gene, *mec*(A), found on a large mobile genetic element, called staphylococcal chromosomal cassette *mec* (SCC*mec*) (Alekshun and Levy, 2007). (PBP)-2a has a decreased binding affinity for β-lactam antimicrobials and requires higher penicillin concentrations for inactivation of bacteria than PBP's. PBP are penicillin-binding proteins of *S. aureus* that are capable of catalyzing the transpeptidation reaction, which is the reaction inhibited by β-lactam antimicrobials (Reed *et al.*, 2011). There are currently 11 different SCC*mec* types recognized based on the combination of the cassette chromosome recombinase (*ccr*) gene complexes and *mec*(A) regulatory genes, *mec*(I) and *mec*(RI) (Ito *et al.*, 2009). The recombinase genes of SCC*mec* allow the cassette to transfer into methicillin susceptible *S. aureus* (MSSA) and lead to emergence of MRSA. The existence of the *mec*(A) gene in MRSA is the specific molecular trait that differentiates MRSA from MSSA. The first three SCC*mec* types mainly cause nosocomial blood stream infections while type IV, V is mainly responsible for community acquired infections (Martins and Cunha, 2007). ST1, ST8, ST30, ST59, ST80 and ST93 are the major ST types associated with CA-MRSA (Vanderhaeghen *et al.*, 2010)

Although genotypic methods of detection of methicillin resistance are widely applied in epidemiological studies and promise high sensitivity and specificity, not all laboratories get these resources. Therefore, phenotypic methods recommended by National Committee for Clinical

Laboratory Standards (NCCLS) and Clinical and Laboratory Standards Institute (CLSI) are required. The methods include the determination of MIC broth/agar dilution, disc diffusion, screening on Mueller-Hinton (MH) agar with 4% NaCl and 6µg oxacillin, and cefoxitin disk diffusion test (Martins and Cunha, 2007).

Epidemiology of MRSA in meat

Together with importance of MRSA as a hospital and community pathogen, it is well-known for food intoxication. Food poisoning from MRSA have been reported once in the USA due to consumption of baked pork meat and coleslaw contaminated with an MRSA producing entertoxin C. It was assumed that the source of contamination was food handler. Further, outbreak strain was of hospital origin (Jones *et al.*, 2002). So, MRSA transmission from food handlers can be an important route of transmission of MRSA to the community. One example of this case is the outbreak initiated by transmission of MRSA by MRSA colonized health care worker involved in food preparation in Dutch hospital outbreak (Kluytmans *et al.*, 1995). Therefore, food can be an excellent way for introduction of antimicrobial resistant microorganisms in general population and in immuno-comprised people. It may transfer antimicrobial-resistant bacteria to the intestinal tract of humans where exchange of resistance genes between non-pathogenic and pathogenic or opportunistic bacteria occurs (Marshall *et al.*, 2009). Although, humans and animals are well-known to be reservoirs and carriers of *Staphylococcus aureus* and are able to transmit the pathogen to food, the accurate prevalence of staphylococcal food poisoning is unknown because it is not reportable through active surveillance system such as FoodNet Surveillance System (Williams, 2012).

MRSA has been identified in retail meat worldwide (Pu *et al.*, 2009; Weese *et al.*, 2010; Lim *et al.*, 2010; De Boer *et al.*, 2009), the potential exists for its transmission to humans. Of the various meat products surveyed, pork had the highest contamination rate in the US and Canada (Pu *et al.*, 2009; Weese *et al.*, 2010), as did beef in South Korea (Lim *et al.*, 2010) and poultry in the Netherlands (De Boer *et al.*, 2009). US study found MRSA clone USA300, having MLST type ST8 and *spa* type 008 and carrying SCC*mec* IVa which is a CA-MRSA strain and has widely disseminated in the US as well as worldwide (Larsen *et al.*, 2009). The study in South Korea also observed MRSA from chicken, which demonstrated ST692 by MLST, a type distinct from that isolated in beef and pork. MRSA of suspected human origin was found in chicken meat in Jordan (Quddoumi *et al.*, 2006). In addition to MRSA strain of human origin, Japan study also reported that the strain was capable of producing entertoxin C (Kitai *et al.*, 2005). Despite sample size variations, these studies suggested that MRSA contamination in different meat categories can vary by location and that molecular distinction may exist among MRSA isolates in meat of different origin.

Furthermore, a new multidrug-resistant *S. aureus* strain, ST398, first discovered in 2003, has emerged in the community (Waters *et al.*, 2011; Khanna *et al.*, 2008; Smith and Pearson, 2011), and MSSA ST398 have been reported in retail meat (Waters *et al.*, 2011) in the US, indicating meat as a potential reservoir of MRSA and MSSA ST398. A recent report has investigated human infections by livestock-associated MSSA ST398, suggesting that this strain is emerging in the community (Jimenez *et al.*, 2011). Importantly, infection was described in individuals with no history of contact with livestock, raising concerns about the changing epidemiology of this pathogen. A Dutch study reported 85% prevalence of LA-MRSA in 11.9% positive raw meat samples. Isolation rate was highest for turkey (35.3%) followed by chicken

(16%), veal (15.2%), pork (10.7%), beef (10.6%), lamb and mutton (6.2%), fowl (3.4%) and game (2.2%) (De Boer *et al.*, 2009). It is believed that during slaughtering of MRSA-positive animals, contamination of carcass and the environment may occur resulting in contaminated meat. Therefore, possibility of becoming colonized with MRSA during food processing or consumption exists. Moreover, immune-compromised people may develop invasive disease following the ingestion of contaminated food (Kluytmans, 2010). According to recent report, meat may serve as a vehicle for dissemination of MRSA in community (Ogata *et al.*, 2012). It is clear from aforementioned data that MRSA strains with different genetic background are present in food, posing a potential risk for public health. Elimination of all risk will be impossible as MRSA become established in meat. Therefore, well-designed studies of MRSA in meat, characterization of interspecies transmission, identification of human health risks, and development of control measures will reduce the impact on human health and agriculture.

CoNS serve as Reservoirs of Antimicrobial Resistance

Although being recognized as opportunistic pathogens, CoNS are often associated with a variety of animal and human diseases, such as suppurative disease, arthritis, and urinary tract infection in animals, and skin and soft tissue infections, and bacteremia in humans (Martins and Cunha, 2007) Ubiquitous and resistant to host of antimicrobials, these microorganisms need an immediate attention as they can transfer resistance genes to other pathogens, which reside in the same environment (Marshall *et al.*, 2009). Some CoNS appear to serve as antimicrobial resistance gene reservoirs capable of converting MSSA to MRSA (Zhang *et al.*, 2009). This has public health implications because CoNS colonize both animals and humans, and CoNS can be transmitted from food animals and retail meat to humans.

With regard to prevalence of multidrug resistance at farm level, most studies on antimicrobial-resistant *Staphylococcus* have focused on *S. aureus* (Martins and Cunha, 2007) from food producing animals, whereas less research effort has been put on CoNS, a group of staphylococci that are believed to be a larger reservoir of resistance genes. Animals are natural reservoir of CoNS. Humans can get CoNS infection from animals by animal handling, especially when animals have a very high CoNS load. CoNS in food animals can also contaminate the animal products and enter the food chain, thus pose potential threat to food consumers and handlers. More importantly, methicillin-resistant coagulase negative Staphylococci (MRCoNS) has been isolated worldwide from food animals (Haenni *et al.*, 2011; Kawano *et al.*, 1996; Feßler *et al.*, 2010), including pigs, cows, calves, and chicken, and therefore, may compromise the treatment if animal or human infection occurs. A recent study from Switzerland revealed 48.75% of MRCoNS from livestock, and strikingly, high percentages of CoNS were also resistant to non-β-lactam antimicrobials, suggesting that multidrug-resistant CoNS may become an emerging problem for veterinary medicine and public health (Huber *et al.*, 2011). However, current knowledge is limited regarding the presence of MRCoNS and the resistance of MRCoNS to non-β-lactam antimicrobials, in food animals in the US (Aarestrup, 2004).

Antimicrobial resistance in *S. aureus* isolated from food is widely investigated; however, prevalence of antimicrobial resistance and determinants in CoNS from food remains unrecognized. Recent study on ready-to-eat meat products in Poland found that prevalence of antimicrobial resistance genes in CoNS from meat was higher than *S. aureus* (Podkowik *et al.*, 2012). These findings suggest meat as an important reservoir of antimicrobial resistant CoNS potentially contributing to the emergence of antimicrobial resistance in other bacteria that reside in the same niche. In this study, none of the *S. aureus* possessed *mec*(A) when compared to 35%

mec(A) positive CoNS indicating meat as a larger reservoir of MRCoNS than MRSA. Moreover, 25% of the CoNS harbored at least four genes encoding resistance to clinically used antimicrobials. Since, genes encoding antimicrobial resistance are usually located on mobile genetic elements, therefore, transfer to pathogenic staphylococcal species is possible. Transmission of antimicrobial resistant bacteria and antimicrobial resistant genes to humans via the food chain has already reported (Simeoni *et al.,* 2008). Thus, these commensals with their reservoir of antimicrobial resistance genes, could be contributing to the rising level of multidrug resistance now commonly seen among pathogens that infect humans and animals (Marshall *et al.,* 2009).

CoNS from oropharangeal cavity and rectum/cloacal of a variety of food animals were identified at species level in previous study (Zhang *et al.,* 2009). Figure 1 shows the distribution of CoNS species in animals. This study aimed to type SCC in CoNS concluded that heterogenous SCC existed in CoNS of diverse genetic background and horizontal transfer of SCC*mec* occurred in the animal production environment. To increase our understanding on the CoNS as reservoir of antimicrobial resistance genes and potential for gene transfer, the manuscript presented here will investigate the antimicrobial resistance phenotypes and genotypes of these isolates from animals.

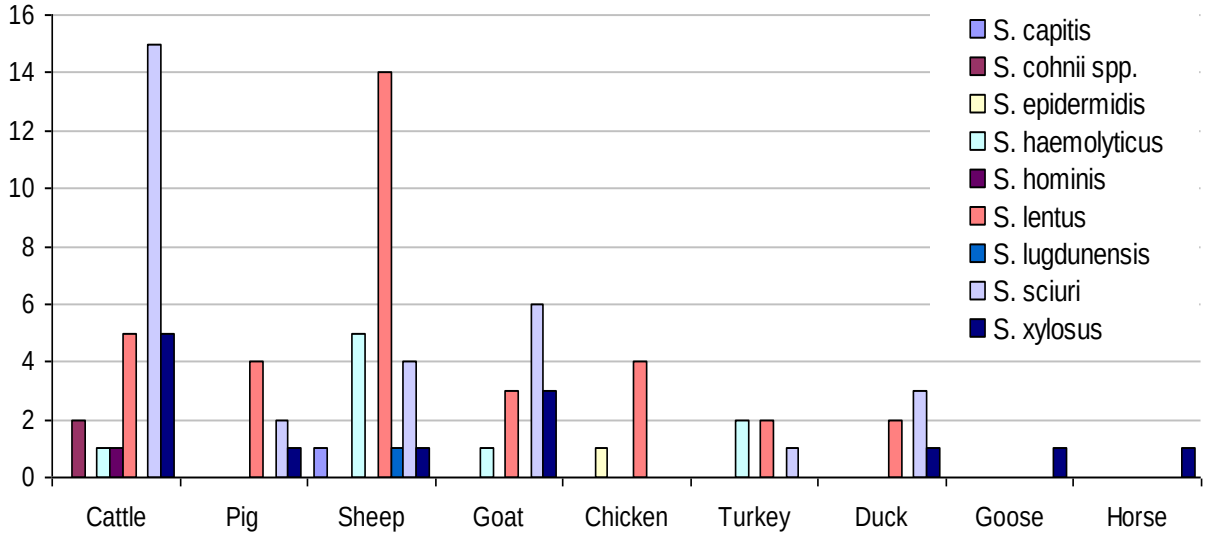


Figure 1. Distribution of coagulase-negative staphylococci (CoNS) in animals

Role of MRCoNS in the emergence of MRSA

The significance of MRCoNS is also reflected by its potential contribution to the emergence of MRSA by transmitting *mec*(A) to MSSA, especially because CoNS is a larger reservoir of SCC*mec* elements as compared to *S. aureus* (Zhang *et al.*, 2009). Although the theory of horizontal transfer of *mec*(A) is well established, the transfer mechanism has yet to be discovered. Investigating the origin/reservoir of *mec*(A) gene is important for understanding the emergence of MRSA (Tsubakishita *et al.*, 2010). It is believed that *mec* genes and *ccr* genes were brought together in CoNS from unknown source and then the genes were transferred into *S. aureus* (Hanssen and Ericson Sollid, 2006). *S. epidermidis* carrying SCC*mec* type IV has been associated with the conversion of MSSA to MRSA (Hiramatsu *et al.*, 2001). SCC*mec* type IV, which is relatively smaller in size when compared to other SCC*mec* types, has been detected in

different genetic backgrounds and is expected to transfer easily among staphylococci (Fey *et al.*, 2003). Further, *S. epidermidis* shows 98-99% homology to SCC*mec* IV_a of *S. aureus* (Barbier *et al.*, 2010).

mec(A) gene homologues have been found in *S. sciuri* and *S. vitulinus*. Moreover, it has been shown that *mec*(A) homologue present in *S. fleuretti* showed 99 to 100% sequence homology with the *mec*(A) gene present in MRSA (Tsubakishita *et al.*, 2010). These findings suggest that direct precursor of the *mec*(A) for MRSA is present in the member of *S. sciuri* group, *S. fleuretti* (Tulinski *et al.*, 2012). Since, *S. fleuretti* is a commensal inhabitant of animals, it strongly indicates the emergence of SCC*mec* element in animal host of *Staphylococcus* species. Moreover, *S. scuiri* group which consist of *S. scuiri*, *S. vitulinus*, *S. lentus* and *S. fleuretti* are usually isolated from variety of animals and food products of animal origin and not isolated from humans (Tsubakishita *et al.*, 2010). This argument is further supported by the fact that MRCoNS is larger reservoir of *mec*(A) than MRSA (Tulinski *et al.*, 2012).

A recent study has claimed that livestock associated MRSA CC398 originated as MSSA in humans. According to this study, the jump of CC398 from humans to livestock was accompanied by the acquisition of tetracycline and methicillin resistance which raises concern about the bidirectional zoonotic exchange and potential public health risks of emergence of antimicrobial resistant bugs due to widespread antimicrobial use in food animal production (Price *et al.*, 2012). The studies to understand the molecular composition of MRCoNS in food production environment are very limited worldwide. MRCoNS of animal origin are being characterized with respect to their SCC composition (Zhang *et al.*, 2009) and MRCoNS has been recovered from minced meat (Huber *et al.*, 2011). However, there is no report on the SCC*mec* types of MRCoNS from meat till date.

Antimicrobial phytochemicals against MRSA

The discovery of antimicrobials eradicated the infections that once ravaged the mankind. However, their liberal use has led to development of multidrug-resistant pathogens (Hemaiswarya *et al.*, 2008). Following the introduction of penicillin, *S. aureus* has developed resistance in early 1940s. Until then, incidence of infections due to these drug resistant microbes is a global problem posing a public health concern. The emergence of these microbes is not only causing treatment failure, but the effectiveness of current drugs is decreasing (Simoes *et al.*, 2009).

With regard to MRSA, the prevalence rate and incidence of infections are increasing worldwide (Crum *et al.*, 2006). MRSA infections are no longer restricted in hospitals (HA-MRSA) and there has been an increase in community acquired MRSA infections. Currently, there are considerable reports on the progress of resistance to the last line of defense such as vancomycin. With the emergence of vancomycin-resistant MRSA, which in future may become a serious problem in treatment of MRSA infections, it is necessary to identify new alternatives. The development of bacterial resistance is unavoidable as it is an important aspect of evolution of bacteria. There is a continuous need to identify new sources of antimicrobials due to existence and continuous evolution of resistant microbes (Wright and Sutherland, 2007).

Phytochemicals, plant derived compounds are well-recognized for their therapeutic power. The interest in using phytochemicals for the treatment of microbial infection started gaining momentum in the late 1990's with the increased inefficacy of conventional antimicrobials (Simoes *et al.*, 2009). Plants produce enormous variety of antimicrobials classified as phytoalexins such as terpenoids, glycosteroids, flavonoids and polyphenols. Their

antimicrobial potential when used alone, and as synergistic with less effective antimicrobial have been confirmed (Abreu *et al.*, 2012).

Despite the abundant literature on the antimicrobial potential of plant compounds, none of the phytochemicals have successfully been exploited for clinical use as antimicrobial. Their limited application is attributed to weak antimicrobial efficacy when compared to common antimicrobials from bacteria or fungi. However, they work very efficiently in synergy as plants exploits synergy of antimicrobials to fight infections. One of the examples of this phenomenon is the combined action of berberine and 5ˈmethoxyhydnocarpin in berberry plants. Berberine that intercalates into DNA is extruded by multidrug resistant pumps. These pumps are blocked by 5ˈmethoxyhydnocarpin (Lewis and Ausubel, 2006; Stermitz *et al.*, 2000).

The emergence of antimicrobial-resistant *Staphylococcus* in the environment poses important public health consequences. Along with other strategies to address antimicrobial-resistant infections, natural compounds may be of value as a novel means for controlling MRSA by increasing the effectiveness of currently available antimicrobials. Antimicrobial combinations of natural antimicrobials with previously applied antimicrobials may decrease the Minimum Inhibitory Concentration (MIC) of traditional antimicrobials against MRSA. Furthermore, combination therapy may be used to deliver a broad-spectrum coverage, prevents the emergence of resistant mutants and obtains a synergy between both antimicrobial agents (Eliopoulos and Moellering, 1996).

Several studies explored the antimicrobial activities of phytochemicals to increase the effectiveness of antimicrobials for which resistance is already established. Antimicrobial activity of berberine in combination with ampicillin and oxacillin against MRSA has revealed that it has

potential to restore the effectiveness of β-lactam antimicrobials against MRSA (Yu *et al.*, 2005) Epigallocatechin gallate from green tea has been shown to work synergistically with β-lactam antimicrobials, since, both of them attacked the same target site peptidoglycan present on the cell wall (Zhao *et al.*, 2001; Hu *et al.*, 2001). A Polyphenol corilagin is found to reduce the MIC of β-lactam by inhibition of PBP2a activity or production. Table 3 lists the synergy studies between phytochemicals and commercial antimicrobials against MRSA. Recently, the role of natural compounds to increase effectiveness of antimicrobials against drug resistant bacteria has been explored and synergistic effect of current antimicrobials and plant derived compounds was observed (Palaniappan and Holley, 2010). However, this study fails to include MRSA, the pathogen which needs immediate attention. Exploring the natural antimicrobials as antimicrobial adjuncts for MRSA is an approach which can extend the life of successful antimicrobial drugs (Wright and Sutherland, 2007).

Table 3. Synergism studies between phytochemicals and antimicrobials against MRSA

Phytochemical	Antimicrobial	References
Berberine	AMP, OXA	(Yu *et al.*, 2005)
Totarol	MET	(Muroi and Kubo, 1996)
Epigallocatechin	AMP, PEN, CAR, TET	(Hemaiswarya *et al.*, 2008)
Tea Catechin	OXA	(Takahashi *et al.*, 1995)
α-Mangostin	VAN, GEN	(Sakagami *et al.*, 2005)
Corilagin	OXA	(Shimizu *et al.*, 2001)
Baicalin	β-lactam	(Liu *et al.*, 2000)
Tellimagrandin I	β-lactam	(Shiota *et al.*, 2000)
Rugosin B	β-lactam	(Shiota *et al.*, 2000)
Pomegranate extract	CIP, CHL, GEN, AMP, TET, OXA	(Braga *et al.*, 2005)
Sophoraflavanone G	VAN, MET, GEN, LEVO	(Sakagami *et al.*, 1998)

Antimicrobial abbreviations: AMP, ampicillin; β-lactam, β-lactam antimicrobials; CAR, Carbapenems; CHL: chloromphenicol; CIP, ciprofloxacin; GEN, gentamicin; LEVO, levofloxacin; MET, methicillin; OXA, oxacillin; PEN, penicillin; TET, tetracycline; VAN, vancomycin

Three things may occur when two antimicrobials are used in combination. They may be additive, synergistic or exhibit antagonism. Additivity is defined as "combination where combined effect is equal to the sum of effects observed with two agents tested separately or equal to that of the most active agent". Overall the antimicrobial activity is neither enhanced nor reduced in the presence of other compound. Synergism is defined as "combination where the antimicrobial effect observed with the combination is greater than the sum of the effects observed with the two compounds independently". On the contrary, antagonism occurs when

there is reduction antimicrobial activity of a compound in the presence of second the antimicrobial agent (Davidson and Parish, 1989; Barry, 1976).

To accurately predict the synergy between commercially available antimicrobials and a phytochemicals, several methods including checkerboard, time-kill, and E test method are mentioned in literature. Checkerboard is widely used and relatively easy to perform in microtitre plate, whereas time-kill assay is labor intensive and time-consuming process (White *et al.*, 1996). In checkerboard, each row and column in the plate contain the same concentration of the first and the second antimicrobial, while the concentration in subsequent row/column is half this value. MIC for the combination is a drug combination in which the growth is completely inhibited. In comparison, time-kill method estimates the bactericidal activity of the antimicrobial alone as well as different concentration of combination of antimicrobial as a function of time. The tubes containing the antimicrobials and their combinations are inoculated with 5×10^5 CFU/ml of bacteria and enumerated by plating on agar plates at fixed interval starting from 0 to 24 hr of incubation. Other method for synergistic evaluation is E test, which can be routinely performed in clinical microbiology (Hemaiswarya *et al.*, 2008).

For all the above methods, Fractional Inhibitory Concentration depicts the interaction between two antimicrobial agents. FIC for each antimicrobial is calculated by dividing MIC of compound present in combination by MIC of compound alone to inhibit microorganism. FIC index is the sum of these two individual FIC values and when this value is equal to or less than 0.5, the combination is termed synergistic. When FIC index value is in between 0.5-1.0 and 1.0-4.0, it indicates additivity and indifference respectively. However, if the value is more than 4 then it specifies antagonism (Mulyaningsih *et al.*, 2010). In general, combined FIC near 1 indicates additivity, <1 indicates synergy and >1 indicates antagonism (Davidson and Parish,

1989). Isobologram method may be applied to represent these observations graphically. The curve is concave in synergism and convex in antagonism (Hemaiswarya *et al.*, 2008).

There is plenty of hope for the phytochemicals to be used in combination with antimicrobials as anti-infective drug. The safe consumption of many phytochemicals indicates their low toxicity. Thus there is a potential to reduce undesirable side effect of antimicrobials on animal and human health by combining the synthetic substances by negligibly toxic and highly specific phytochemical antimicrobials (Sibanda and Okoh, 2007). Although, several reports are available that describe the antimicrobial behavior of phytochemicals, however, identification of compounds with resistance modifying action is of particular interest. With the advent of resistance to almost all commercially available antimicrobials, in vitro screening procedures for drug combination should be speed up to achieve breakthrough in combination therapy (Hemaiswarya *et al.*, 2008).

CHAPTER 1

MULTIDRUG-RESISTANT COAGULASE-NEGATIVE STAPHYLOCOCCI IN FOOD ANIMALS

Introduction

Although being recognized as opportunistic pathogens, coagulase-negative staphylococci (CoNS) are often associated with a variety of animal and human diseases, such as suppurative disease, arthritis, and urinary tract infection in animals (Zhang *et al.*, 2009), and skin and soft tissue infections and bacteremia in humans (Martins *et al.*, 2007). Animals are natural reservoir of CoNS. Human can get CoNS infection from animals by animal handling. CoNS in food animals can also contaminate the animal products and enter the food chain, thus pose potential threat to food consumers and handlers.

More importantly, methicillin-resistant CoNS (MRCoNS) have been isolated worldwide from food animal (Weigel *et al.*, 2007; Hanssen *et al.*, 2004; Volokhov *et al.*, 2003), including pigs, cows, calves, and chicken, and therefore, may compromise the treatment if animal or human infection occurs. Interestingly, resistance to non-β-lactam antimicrobials, such as erythromycin, tetracycline, clindamycin, ciprofloxacin, sulphamethoxazole/trimethoprim, is common in MRCoNS (Simeoni *et al.*, 2008; Huber *et al.*, 2011). A recent study from Switzerland revealed 48.2% of MRCoNS from livestock and high percentages of CoNS were also resistant to non-β-lactam antimicrobials, suggesting that multidrug-resistant CoNS may become an emerging problem for veterinary medicine and public health (Huber *et al.*, 2011). However, most studies on CoNS in animals have been performed in Europe. There are limited data on CoNS in US food animals as well as resistance of CoNS to non-β-lactam antimicrobials.

The present study aimed at understanding CoNS of animal origin as a reservoir of multidrug resistance.

Materials and methods

Bacterial strains

A total of 87 CoNS from a variety of agricultural animals was selected from a collection of CoNS that were potentially resistant to β-lactam antimicrobials. Staphylococcal species identification has been performed in a previous study (Zhang *et al.*, 2009) and the isolates consisted of *S. lentus* (33), *S. sciuri* (30), *S. xylosus* (12), *S. haemolyticus* (9), and one each of *S. capitis, S. epidermidis, and S. hominis*. CoNS-carrying animals included cattle (n = 27), sheep (n = 25), goats (n = 13), pigs (n=7), chicken (n = 5), turkey (n = 5), duck (n = 3), geese (n=1) and horses (n=1).

Antimicrobial susceptibility testing of CoNS

Antimicrobial susceptibility of the 87 CoNS was determined using the Sensititre Antimicrobial Susceptibility System (Trek Diagnostic Systems, Westlake, OH) and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Sutcliffe *et al.*, 1996). Ampicillin, cefoxitin (6 μg/ml), chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, linezoid, livofloxacin, moxifloxacin, nitrofurantoin, oxacillin, penicillin, quinupristin-dalfopristin (Q/D), rifampin, streptomycin, tetracycline, tigecycline, sulfamethoxazole/trimethoprim, and vancomycin were tested. *Staphylococcus aureus* ATCC 29213 was used as the quality control microorganism.

Molecular determination of antimicrobial resistance

Genomic DNA template used for PCR was extracted by a boiling method as previously described (Zhang *et al.*, 2005). *mec*(A) PCR (Perazzi *et al.*, 2006) was performed in all 87 CoNS, whereas 10 genes conferring resistance to macrolides-lincosamide-streptogramin B (MLS_B) group and tetracycline were tested only on isolates demonstrating the resistance phenotypes. The genes tested were *erm*(A), *erm*(B), *erm*(C), *vat-1*, *vat-2*, *vat-3*, *vga*(A), *vga*(B), *tet*(K), and *tet*(M) (Volokhov *et al.*, 2003; Sutcliffe *et al.*, 1996; Soltani *et al.*, 2000; Weigel *et al.*, 2007). In addition, tetracycline-resistant isolates were also examined for the presence of Tn*916*, a conjugative transposon (Soge *et al.*, 2008).

Conjugation experiments

Tetracycline-resistant CoNS were used as donor strains in conjugation experiments to study tetracycline resistance gene transfer. *Enterococcus faecalis* JH2-2 (rif^r fus^r) was used as the recipient strain. Conjugation was performed by the filter mating method (Agersø *et al.*, 2006) with modifications. Briefly, overnight cultures of the donor strains grown in Brain Heart Infusion (BHI) broth (Difco, Sparks, MD) containing tetracycline (16 μg/mL) and recipient grown in BHI containing fusidic acid (50 μg/mL) and rifampicin (50 μg/mL) were mixed (ratio, 1:1) in BHI broth. The mixture was then placed on a 0.45-μm-pore-size filter and incubated on BHI agar plates (Difco) at 37°C overnight. The filter was washed and vortex- mixed in BHI broth. The mating mixture was spread onto BHI agar containing a combination of tetracycline (16 μg/mL), fusidic acid (50 μg/mL), and rifampicin (50 μg/mL). Up to three potential transconjugants were purified on BHI agar containing appropriate antimicrobials and resistance gene transfer by conjugation was confirmed by PCR.

Results

Antimicrobial susceptibility profiles of CoNS

All 87 CoNS showed resistance to oxacillin, whereas only 47 were able to grow at 6 µg/ml of cefoxitin. Resistance to the other two β-lactam antimicrobials were also high, 79.3 % to ampicillin and 91.9 % to penicillin. Fifty-nine isolates were resistant to tetracycline (67.8%), followed by 32 to erythromycin (36.8%), 24 to clindamycin (27.6%), and 13 to quinupristin-dalfopristin (14.9%). Resistance to other antimicrobials, such as chloromphenicol, ciprofloxacin, gentamicin, and sulfamethoxazole-trimethoprim, were also identified, though at low prevalence rates. All 87 CoNS were resistant to at least one β-lactam antimicrobial, among which 16 were resistant to β-lactam only and 24 were resistant to β-lactam and one other antimicrobial. The remaining 47 isolates (54%) were resistant to at least three antimicrobial classes and defined as multidrug resistance, including 22 of 33 *S. lentus*, 15 of 30 *S. sciuri*, 1 of 12 *S. xylosus*, and 9 of 9 *S. haemolyticus*. Twenty-one isolates demonstrated resistance to 3 classes, followed by 14 to 4 classes, 6 each to 5 and 6 classes. The common resistance profiles were β-lactam only (16), β-lactam and tetracycline (16), β-lactam, erythromycin, and tetracycline (13), as well as β-lactam, clindamycin, erythromycin, and tetracycline (7) (Figure 1).

All 9 *S. haemolyticus* were β-lactam resistant, whereas prevalence of resistance to ampicillin, penicillin, and oxacillin in *S. lentus*, *S. sciuri*, and *S. xylosus* ranged from 72.7% to 100% (Table 4). Only 1 of 9 *S. haemolyticus* was erythromycin resistant, in comparison with all but one *S. xylosus* being resistant. While high prevalence of resistance was observed in *S. xylosus* to β-lactam, erythromycin, and tetracycline, all 12 *S. xylosus* were susceptible to other antimicrobials tested. A total of 13 CoNS, including 6 each of *S. haemolyticus* and *S. lentus* and 1 *S. sciuri*, were resistant to quinopristin-dalfopristin (Q/D), an antimicrobial that can be used to

treat methicillin-resistant Staphylococcal infection (Qin *et al.*, 2011). The 13 Q/D-resistant CoNS were recovered from 1 cattle, 2 chicken, 7 sheep, and 3 turkey samples.

There was no clear distinction in regards to the prevalence of β-lactam, tetracycline, and macrolide resistance among animal groups. Multidrug-resistant CoNS were recovered from all animal species except for a goose and a horse. Eighty percent each of CoNS from chicken and turkey were multidrug resistant, followed by 66.7% from duck, 61.5% from goats, 60% from sheep, 57.1% from pigs, and 37% from cattle (Figure 2).

Table 4: Prevalence of common antimicrobial resistance in *S. lentus, S. sciuri, S. xylosus, and S. haemolyticus*

Antimicrobials	No. of antimicrobial-resistant CoNS (%)			
	S. haemolyticus (N=9)	*S. lentus* (N=33)	*S. sciuri* (N=30)	*S. xylosus* (N=12)
AMP	9 (100)	24 (72.7)	24 (80)	11 (91.6)
OXA	9 (100)	33 (100)	30 (100)	12 (100)
PEN	9 (100)	28 (84.8)	29 (96.6)	12 (100)
ERY	1 (11.1)	14 (42.4)	15 (50)	11 (91.6)
TET	5 (55.5)	24 (72.7)	20 (66.6)	10 (83.3)
CHL	0 (0)	4 (12.1)	1 (3.3)	0 (0)
CIP	5 (55.5)	0 (0)	1 (3.3)	0 (0)
CLI	7 (77.7)	8 (24.2)	8 (26.6)	0 (0)
GEN	5 (55.5)	1 (3)	1 (3.3)	0 (0)
SYN	6 (66.6)	6 (3)	1 (3.3)	0 (0)
SXT	0 (0)	1 (3)	4 (13.3)	0 (0)

Antimicrobial abbreviations: AMP: ampicilln; CHL: Chloromphenicol; CIP: ciprofloxacin; CLI: clindamycin; ERY: erythromycin; GEN: gentamicin; OXA: oxacillin; PEN: pencillin; SYN: quinupristin-dalfopristin; SXT: sulphamethoxazole/trimethoprim; TET: tetracycline

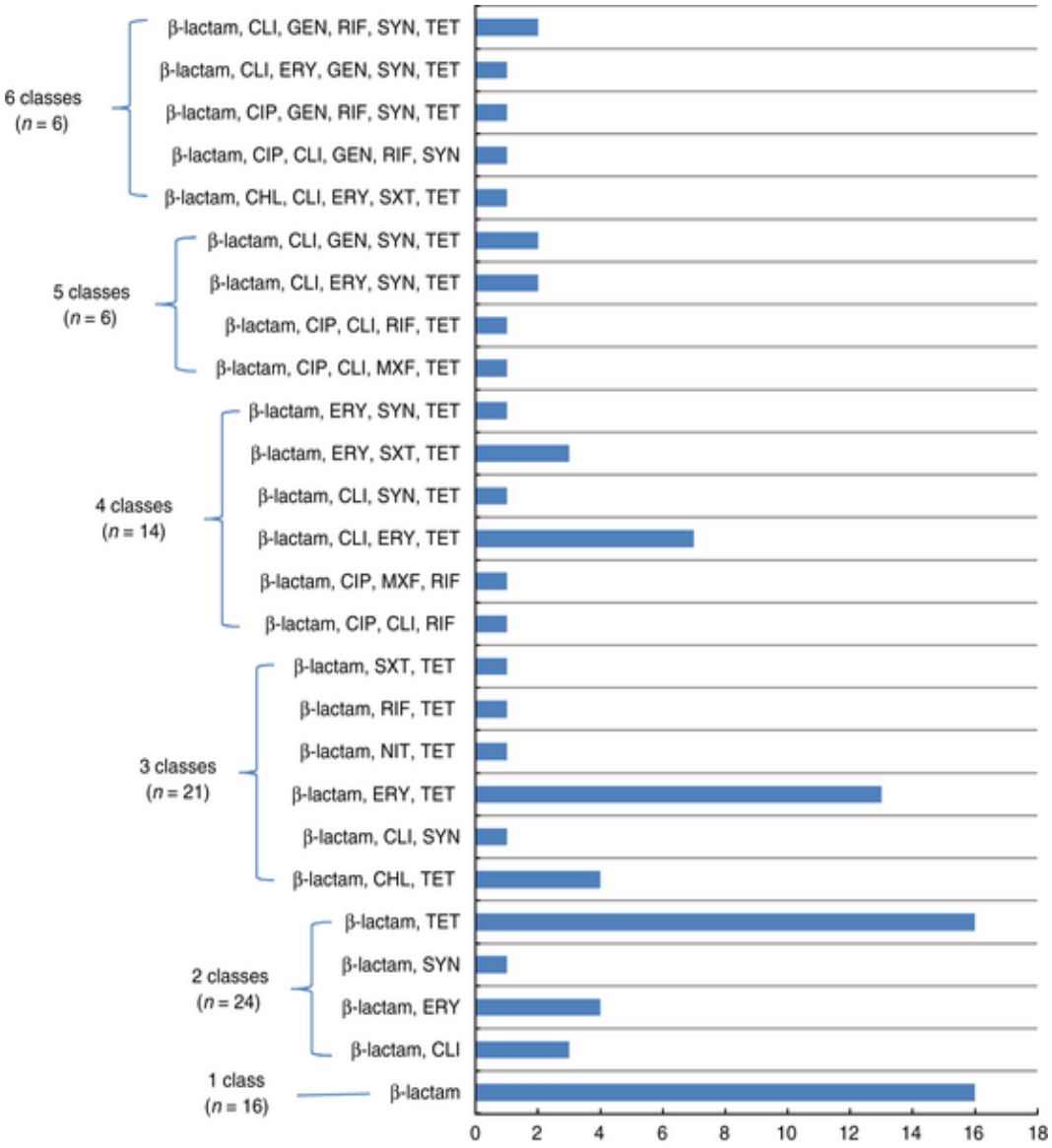


Figure 2: Resistance of CoNS to various numbers of antimicrobial classes in this study

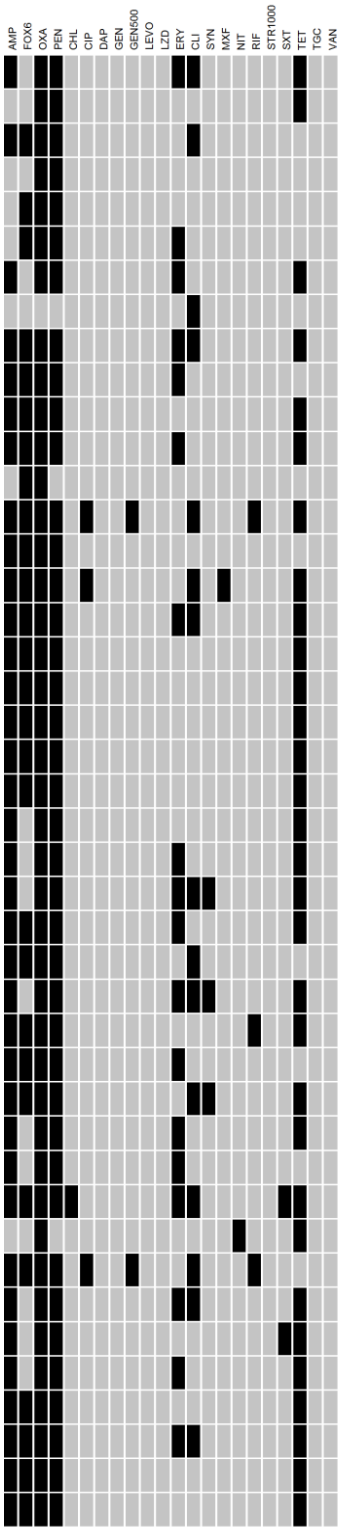
Antimicrobial abbreviations: β-lactam: ampicilln/cefoxitin/oxacillin/pencillin; CHL: Chloromphenicol; CIP: ciprofloxacin; CLI: clindamycin; ERY: erythromycin; GEN: gentamicin; MXF: moxifloxacin; NIT: nitrofurantoin; RIF: rifampin; SYN: quinupristin-dalfopristin; SXT: sulphamethoxazole/trimethoprim; TET: tetracycline

Distribution of antimicrobial resistance genes and Tn*916*

mec(A) was detected in 60 of 87 CoNS (67.8%). *erm*(A), *erm*(B), *erm*(C), and *vga*$(A)_{LC}$, were detected in 31 of 47 isolates that were resistant to clindamycin, erythromycin, and/or Q/D. The 16 remaining isolates, including 13 resistant to erythromycin, did not carry any of the genes tested and were comprised of 8 *S. scuiri*, 5 *S. lentus*, 2 *S. haemolyticus and* 1 *S. epiderdimis*. Moreover, three isolates from cattle, turkey, and chicken (IDs 65, 79, and 86) which were resistant to all MLS antimicrobials carried at least three resistance genes tested. Of 32 erythromycin-resistant isolates, *erm*(A) predominated and was detected in 17 (53.1%) isolates, followed by *erm*(C) (25%) and *erm*(B) (21.9%). With regard to the 24 clindamycin-resistant isolates, *erm*(B) and *erm*(A) were detected in 12 (50%) and 7 (29.2%) isolates, respectively. Q/D resistance genes, *vga*$(A)_{LC}$ and *erm*(B), were both recovered from 9 of 13 (69.2%) Q/D-resistant CoNS (Figure 3).

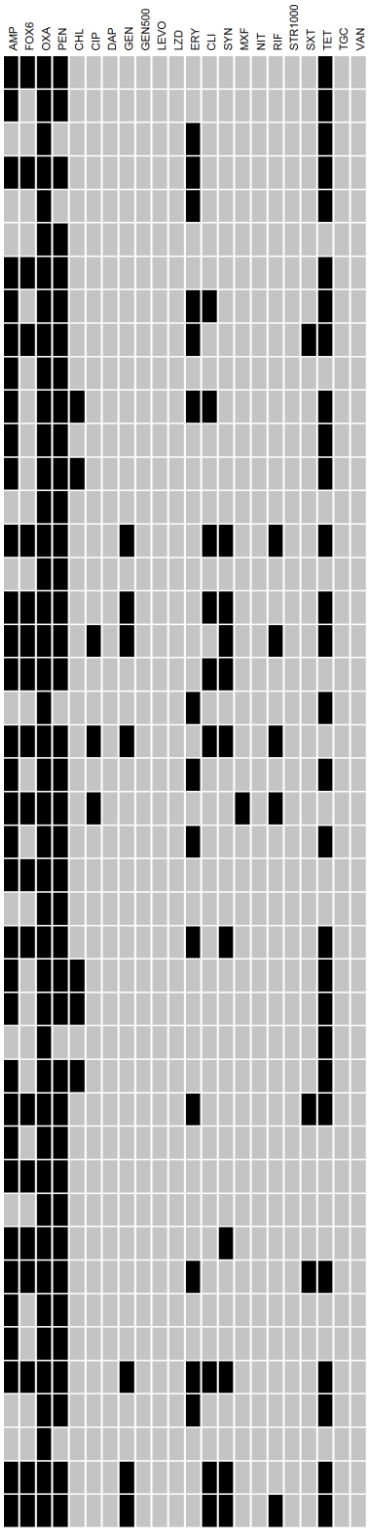
tet(M) was carried by 36 of 59 tetracycline-resistant CoNS, among which 31 also had Tn*916*. *tet*(K) was detected in 27 isolates. Fourteen isolates contained both *tet*(K) and *tet*(M). Furthermore, tetracycline resistance was conjugatively transferable in 11 of 59 (17.74%) tetracycline-resistant CoNS, in which 6 were *S. scuiri*, 4 were *S. lentus*, and 1 was *S. haemolyticus*. All transconjugants were positive for *tet*(M) and Tn*916*.

Antimicrobials



Strain ID	Species	Met-r	Tet-r	MLSb-r	Streptogramin-r
Cattle10#	S. sciuri	mec(A)	tet(K), tet(M), Tn916	erm(A)	
Cattle11	S. sciuri		tet(M), Tn916		
Cattle12	S. lentus				
Cattle13	S. sciuri				
Cattle14	S. sciuri	mec(A)			
Cattle15	S. sciuri	mec(A)			
Cattle16#	S. sciuri	mec(A)	tet(M), Tn916	erm(A)	
Cattle17	S. hominis			erm(B)	
Cattle18#	S. sciuri				
Cattle19	S. sciuri				
Cattle21	S. sciuri				
Cattle22#	S. sciuri		tet(M),Tn916		
Cattle23	S. sciuri				
Cattle24#	S. sciuri	mec(A)	tet(M), Tn916	erm(B)	
Cattle25	S. lentus				
Cattle27#	S. haemolyticus	mec(A)	tet(M), Tn916		
Cattle28#	S. sciuri				
Cattle57	S. xylosus	mec(A)	tet(K)		
Cattle59	S. xylosus	mec(A)	tet(K)		
Cattle60	S. xylosus	mec(A)	tet(K)		
Cattle61	S. xylosus	mec(A)	tet(K)		
Cattle62	S. lentus	mec(A)	tet(K)		
Cattle63	S. sciuri	mec(A)	tet(K)		
Cattle64#	S. xylosus	mec(A)	tet(K), tet(M), Tn916	erm(A), erm(C)	
Cattle65#	S. lentus	mec(A)	tet(K), tet(M), Tn916	erm(A), erm(B), erm(C)	
Cattle66#	S. sciuri	mec(A)		erm(A), erm(C)	
Cattle9	S. lentus			erm(B)	
Chicken79#	S. lentus	mec(A)	tet(M)	erm(A), erm(C)	vga(A)LC
Chicken85#	S. lentus	mec(A)	tet(K), tet(M), Tn916		
Chicken88	S. epidermidis	mec(A)			
Chicken89#	S. lentus	mec(A)	tet(K), tet(M), Tn916		vga(A)LC
Chicken90#	S. lentus	mec(A)	tet(K)		
Duck78	S. sciuri	mec(A)	tet(K)		
Duck83#	S. sciuri		tet(M), Tn916		
Duck94#	S. lentus		tet(M), Tn916		
Goat1#	S. haemolyticus			erm(B)	
Goat2#	S. sciuri		tet(M), Tn916		
Goat3#	S. sciuri	mec(A)	tet(M), Tn916		
Goat4#	S. sciuri	mec(A)	tet(M), Tn916	erm(A), erm(C)	
Goat5	S. sciuri	mec(A)	tet(M), Tn916		
Goat53#	S. sciuri	mec(A)	tet(M), Tn916	erm(A), erm(B)	
Goat54	S. xylosus	mec(A)	tet(K), tet(M), Tn916		
Goat55	S. sciuri	mec(A)			

Antimicrobials



Strain ID	Species	Met-r	Tet-r	MLSb-r	Streptogramin-r
Goat56	S. xylosus	mec(A)	tet(K), tet(M), Tn916		
Goat6	S. xylosus	mec(A)	tet(M), Tn916		
Goat67#	S. lentus	mec(A)	tet(M), Tn916	erm(A), erm(C)	
Goat7#	S. lentus	mec(A)	tet(M), Tn916	erm(A)	
Goat8#	S. lentus		tet(K), tet(M), Tn916	erm(A)	
Goose81	S. xylosus	mec(A)			
Horse72	S. xylosus		tet(K)		
Pig68#	S. lentus	mec(A)	tet(K)	erm(A), erm(B)	
Pig73#	S. sciuri	mec(A)		erm(A), erm(C)	
Pig74	S. sciuri	mec(A)			
Pig75#	S. lentus	mec(A)			
Pig76	S. xylosus	mec(A)	tet(M)		
Pig77#	S. lentus	mec(A)			
Pig82	S. lentus				
Sheep29#	S. haemolyticus		tet(M), Tn916	erm(B)	vga(A)LC
Sheep30	S. lentus	mec(A)			
Sheep31#	S. lentus	mec(A)	tet(K)	erm(B)	vga(A)LC
Sheep32#	S. haemolyticus	mec(A)	tet(M), Tn916	erm(B)	vga(A)LC
Sheep33#	S. haemolyticus				vga(A)LC
Sheep34#	S. lentus	mec(A)	tet(M), Tn916	erm(B)	
Sheep36#	S. haemolyticus	mec(A)			
Sheep37#	S. lentus	mec(A)	tet(K), tet(M), Tn916	erm(A)	
Sheep38#	S. haemolyticus				
Sheep39#	S. lentus	mec(A)			
Sheep40	S. lentus	mec(A)	tet(M)		
Sheep41	S. capitis				
Sheep42#	S. lentus	mec(A)	tet(K)	erm(B)	vga(A)LC
Sheep43#	S. lentus	mec(A)	tet(K), tet(M)		
Sheep44#	S. lentus		tet(K)		
Sheep45	S. lentus	mec(A)	tet(K)		
Sheep46#	S. lentus	mec(A)	tet(K)		
Sheep47#	S. lentus	mec(A)	tet(K), tet(M), Tn916	erm(A), erm(B)	
Sheep48	S. lentus	mec(A)			
Sheep49	S. xylosus	mec(A)			
Sheep50	S. sciuri	mec(A)			
Sheep51	S. lentus	mec(A)		erm(B)	vga(A)LC
Sheep52#	S. sciuri	mec(A)		erm(A)	
Sheep69	S. sciuri	mec(A)			
Sheep80	S. sciuri	mec(A)			
Turkey86#	S. haemolyticus	mec(A)	tet(M), Tn916	erm(A), erm(B), erm(C)	
Turkey87#	S. lentus	mec(A)	tet(K), tet(M)		
Turkey91	S. lentus				
Turkey92#	S. haemolyticus		tet(M), Tn916	erm(B)	vga(A)LC
Turkey93#	S. sciuri		tet(K), tet(M), Tn916	erm(A), erm(B)	

Figure 3: Antimicrobial resistance phenotypes and resistance genes identified in CoNS

***, black, resistance phenotypes; grey, susceptible phenotypes**

Antimicrobial abbreviations: AMP, ampicillin; CHL: chloromphenicol; CIP, ciprofloxacin; CLI, clindamycin; DAP, daptomycin; ERY, erythromycin; FOX6, cefoxitin (6 μg ml^{-1}); GEN, gentamicin; LEVO, levofloxacin; LZD, linezolid; MXF, moxifloxacin; NIT, nitrofurantoin; OXA, oxacillin; PEN, penicillin; RIF, rifampin; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim; SYN, quinupristin/dalfopristin; TET, tetracycline; TGC, tigecycline; VAN, vancomycin

†, # indicates a multidrug-resistant strain.

Discussion

Most studies on antimicrobial-resistant *Staphylococcus* in agriculture have focused on *Staphylococcus aureus* from food or food producing animals, whereas less research effort has been put on CoNS, a group of staphylococci that are believed to be a larger reservoir of antimicrobial resistance genes (Lee, 2003; O'Mahony *et al.*, 2005; Juhász-Kaszanyitzky *et al.*, 2007). To our knowledge, the current study is the first report on multidrug-resistant CoNS from food producing animals in the US. The recovery of 47 multidrug-resistant CoNS (54%) from food animals and the observation of resistance to macrolides, tetracyclines, and Q/D, in addition to β-lactam resistance, suggest food animals as important reservoir of antimicrobial-resistant CoNS.

The four Staphylococcal species, *S. lentus, S. sciuri, S. xylosus,* and *S. haemolyticus*, identified in this study are commonly associated with farm animals (Aarestrup and Schwarz, 2006). The high prevalence of resistance to tetracycline, erythromycin, and clindamycin was not

surprising as compared to previous studies on CoNS in poultry litter in the US (Simjee *et al.*, 2007) and MRCoNS from various sources, including animals, meat, and contact persons, in Switzerland (Huber *et al.*, 2011). When comparing the three studies, we found tetracycline resistance (67.8%) predominated in our study and so did erythromycin resistance (71%) in the Simjee study, whereas no marked difference was observed in the Swiss study in terms of the prevalence of resistance to erythromycin, tetracycline, and clindamycin, ranging from 43.7 to 49% (Huber *et al.*, 2011). This may be an indication of common use of tetracyclines and macrolides in animal production in the US. Recovery of Q/D-resistant CoNS in our study as well as from poultry litter by Simjee, with rates of 14.9% and 13%, respectively, suggests a potential linkage between streptogramin usage on farms and Q/D resistance in this country, although comparison between the US data and data from Switzerland was not possible as Q/D was not tested in the Swiss study.

Because all CoNS included in this study were potentially resistant to β-lactam, it was not surprising that the isolates were resistant to at least one β-lactam antimicrobial. The recovery of high percentages of multidrug-resistant CoNS from most animal categories suggests that animal commensal bacteria are important reservoir of antimicrobial resistance phenotypes. Because animal intestinal environment provides an optimal condition for antimicrobial resistance genes to transfer from commensals to pathogens (Marshall *et al.*, 2009), gene dissemination across species or even genus borders is expected to be common in animal hosts and the extent of antimicrobial resistance can thus be amplified substantially. Multidrug resistance phenotypes were commonly seen in the top three animals carrying CoNS and the prevalence was 37%, 60%, and 61.5%, from cattle, sheep, and goats, respectively, which again is an indication of widespread distribution of multidrug resistance in agriculture, although farm variation in

agricultural practices and level of antimicrobial exposure of bacteria could not be excluded.

The observation of staphylococcal species variation in multidrug resistance is consistent with findings by Huber (Huber *et al.*, 2011) that resistance of CoNS to erythromycin, tetracycline, clindamycin, ciprofloxacin, sulphamethoxazole/trimethoprim, and gentamicin was more common in *S. haemolyticus*, *S. epidermidis*, and *S. sciuri* than that in other species, with *S. haemolyticus* having the highest percentage of non-β-lactam resistance. Another study also found 4 multidrug-resistant *S. haemolyticus* out of 6 total *mec*(A)-positive Staphylococci from clinical animals (van Duijkeren *et al.*, 2004). Together with our results that all 9 *S. haemolyticus* versus only 1 of 12 *S. xylosus* were multidrug resistant, it is reasonable to assume that species variation exists in *Staphylococcus* as to their antimicrobial resistance. *S. haemolyticus* in animals may have stronger public health significance, considering that it is frequently involved in human clinical cases (Huber *et al.*, 2011; van Duijkeren *et al.*, 2004).

erm(A) and *erm*(C) were commonly found in erythromycin-resistant Staphylococci in this study, which is in agreement with previous reports that they were the predominant erythromycin resistance genes in *Staphylococcus* isolated from various sources (Khan *et al.*, 2002; Simjee *et al.*, 2007; Aarestrup *et al.*, 2000; Graham, 2009). Unlike *erm*(A) and *erm*(C), *erm*(B) is less common in *Staphylococcus* than in *Enterococcus* and *Streptococcus*. However, *erm*(B) was identified in 7 erythromycin-resistant CoNS in our study, in contrast to its absence in previous studies (Graham, 2009; Simjee *et al.*, 2007; Aarestrup *et al.*, 2000), suggesting genetic diversity in erythromycin resistance genes in Staphylococci in different geographic locations. Since *erm*(B) can be carried on plasmids, the chromosome, and on transposons (Khan *et al.*, 2002), future examination of the genetic background of *erm*(B) in these isolates would help assess the potential of its dissemination in *Staphylococcus* in animals. Our finding that 13 of 32

erythromycin-resistant isolates had no resistance genes identified was not surprising as compared to previous reports (Simjee *et al.*, 2007), although it could also be due to the limited number of genes tested.

As for streptogramin resistance genes, the *vga*(A) gene detected was confirmed by DNA sequencing as *vga*(A)$_{LC}$. This evolutionary variant of *vga*(A) encodes for an ABC transporter and has been reported in *Staphylococcus* resistant to lincosamides and streptogramin (Novotna and Janata, 2006). Failure to observe clindamycin resistance in some of our *vga*(A)$_{LC}$-positive isolates can be explained by the different phenotypic methods used to determine clindamycin resistance because we used a broth microdilution method whereas agar dilution or disk diffusion were applied in previous studies. In fact, the clindamycin resistance level of *S. haemolytics* carrying *vga*(A)$_{LC}$ reported by Novotna et al. was not very high (Novotna and Janata, 2006; Qin *et al.*, 2011), so borderline resistance phenotypes would not be unexpected if using a different method. In addition, source of isolation may also have impact on antimicrobial resistance phenotypes. All previously reported *Staphylocccus* carrying *vga*(A)$_{LC}$ was from clinical settings where constant antimicrobial selective pressure maintains the resistance phenotypes more effectively than what happens in agriculture.

The two mechanisms of tetracycline resistance reported in *Staphylococcus*, ribosomal protection and active efflux, were identified in this study as evidenced by the presence of *tet*(M), encoding for ribosomal protection, in more than 60% of CoNS, and *tet*(K), encoding for an active efflux, in 45.7% of the isolates. Tn*916*, a conjugative transposon that is often associated with *tet*(M), was detected in most *tet*(M)-positive isolates. The fact that nearly 18% of CoNS could transfer *tet*(M) to *Enterococcus faecalis*, together with Tn*916*, suggests that tetracycline resistance from *Staphylococcus* can transfer to other Gram-positive bacteria that have potential to

cause human diseases. Since antimicrobial resistance genes other than *tet*(M) have been identified on Tn*916* (Del Grosso *et al.*, 2004; Fletcher and Daneo-Moore, 1992; Lancaster *et al.*, 2004), transfer of additional antimicrobial resistance genes should also be expected via this conjugative transposon from a multidrug resistance reservoir.

In conclusion, our data indicate that CoNS in agricultural animals are an important reservoir of multidrug resistance in addition to the resistance to β-lactam antimicrobials and underline the importance of surveillance of multidrug-resistant CoNS in the food production environment. Since our CoNS strains were all resistant to at least one β-lactam antimicrobial, further research is needed as to whether methicillin resistance predisposes CoNS to become multidrug resistant as compared to general CoNS, including the potential linkage, if any, between β-lactam resistance and other resistance phenotypes. Species variation exists in the prevalence of multidrug resistance in Staphylococci. Certain Staphylococcal species, such as *S. haemolyticus*, may have stronger potential to become multidrug resistant and thus require closer research and public health attention.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF *S. AUREUS* ISOLATED FROM RETAIL MEAT IN DETROIT

Introduction

Staphylococcus aureus is one of the most important human pathogen responsible for a wide variety of human ailments including skin, soft tissue and bone infections, pneumonia and bacteremia etc (Lipsky *et al.,* 2007). Antimicrobial resistance in this pathogenic commensal is a serious concern in both community and hospital settings. Methicillin-resistant *S. aureus* (MRSA) is an example of superbugs which has emerged worldwide as a signature of antimicrobial resistance problem. The pathogen has acquired resistance to even vancomycin which is considered the drug of last resort (Marshall *et al.,* 2009).

Methicillin resistance is conferred by the presence of methicillin resistance gene, *mec*(A). Strains negative for *mec*(A) and lacking phenotypic resistance to methicillin are termed as Methicillin Susceptible *S. aureus* (MSSA). MSSA is widely present in nature and may serve as a recipient of antimicrobial resistance genes, including methicillin from other bacteria of human clinical significance such as MRSA or MRCoNS which resides in same niche as MSSA (Hiramatsu *et al.,* 2001). *S. epidermidis mecA* was identical to that identified in a *Staphylococcus aureus* isolate from the same individual in vivo (Wielders *et al.*, 2001). Therefore, it is assumed that MSSA strain acquired the SCC*mec* IV from *S. epidermidis* by horizontal gene transfer leading to emergence of CA-MRSA (Hanssen and Ericson Sollid, 2006).

MRSA has been identified in retail meat worldwide (Pu *et al.,* 2009; Weese *et al.,* 2010; De Boer *et al.*, 2009; Lim *et al.,* 2010), the potential exists for its transmission to humans. Of the

various meat products surveyed, pork had the highest contamination rate in the US and Canada (Pu *et al.*, 2009; Weese *et al.*, 2010), as did beef in South Korea (Lim *et al.*, 2010) and poultry in the Netherlands (De Boer *et al.*, 2009). The study in South Korea also observed MRSA from chicken, which demonstrated ST692 by MLST, a type distinct from that isolated in beef and pork. Despite sample size variations, these studies suggested that MRSA contamination in different meat categories can vary by location and that molecular distinction may exist among MRSA isolates in meat of different origin. Furthermore, a new multidrug-resistant *S. aureus* strain, ST398, first discovered in 2003, has emerged in the community (Khanna *et al.*, 2008; Smith and Pearson, 2011), and MSSA ST398 have been reported in retail meat in the US (Waters *et al.*, 2011), indicating meat as a potential reservoir of MRSA and MSSA ST398. A recent report has investigated human infections by livestock-associated MSSA ST398, suggesting that this strain is emerging in the community (Jimenez *et al.*, 2011). Importantly, infection was described in individuals with no history of contact with livestock, raising concerns about the changing epidemiology of this pathogen. However, studies to explore the molecular epidemiology of *Staphylococcus* spp. in US retail meat are limited.

Meat products and food animals are important environmental reservoir of bacteria. MRSA has been identified in food animals (cows, pigs, and chicken) and animal workers worldwide (De Neeling *et al.*, 2007; Smith *et al.*, 2009). An animal farming-related MRSA clone (ST398), which has gained particular attention as source of infection in animals, was also observed from meat products in the Netherlands (Van Loo *et al.*, 2007; De Boer *et al.*, 2009). Importantly, human epidemic clones of MRSA (USA300 and USA100) have been isolated from retail pork in the US (Pu *et al.*, 2009), and thus a potential transmission of MRSA from retail meat to humans exists.

The objective of this specific aim is to characterize MRSA and MSSA strains recovered from retail meat. The proposed work will improve our understanding on the molecular epidemiology of this pathogen and potential of retail meat to transmit human *Staphylococcal* infections.

Material and Methods

Sample Collection

A total of 289 raw meat samples (76 chicken, 156 beef and 57 turkey) were collected from 30 randomly-selected retail meat stores in Detroit, Michigan during August 2009 to January 2010.

Bacteria Isolation & Confirmation

The meat samples were enriched for 24 hours in tryptic soy broth (TSB) with 10% of NaCl and 1% of sodium pyruvate, followed by the selection of potential *S. aureus* on mannitol salt agar (MSA). *S. aureus* were confirmed by coagulase test and PCR targeting the *Staphylococcus* genus-specific gene and *S. aureus* species-specific gene.

Characterization of MRSA & MSSA isolates

MRSA and MSSA as evidenced by the presence or absence of *mec*(A) by PCR were further characterized by phenotypic and genotypic methods

Antimicrobial Susceptibility Tests

a. **Broth Micro Dilution Method:** Sensititre Antimicrobial Susceptibility System (Trek Diagnostic Systems, Westlake, OH) was used to determine the MIC of antimicrobials on MRSA and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution (Wikler *et al.*, 2006).

Briefly, 96-well plates containing ampicillin, cefoxitin (6µg/ml), chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, linezoid, livofloxacin, moxifloxacin, nitrofurantoin, oxacillin, penicillin, quinupristin-dalfopristin (Q/D), rifampin, streptomycin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole, and vancomycin in different concentration were inoculated with $5x10^5$ CFU/ml in Cation Adjusted Mueller Hilton II Broth (CAMHB) containing 4% NaCl. Further, plates were covered with plate seals and incubated at 35ºC for 24 hours. *S. aureus* ATCC 29213 was used as the quality control microorganism.

b. **Disc Diffusion Method:** Standard Disc Diffusion Method was used to evaluate the antimicrobial susceptibility of MSSA against a range of antimicrobials, including ampicillin, ciprofloxacin, clindamycin, erythromycin, gentamicin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin (Zhang *et al.*, 2010). An inoculum of 1–2 x 10^8 CFU/ml is prepared and inoculated on Mueller Hilton Agar (MHA). The MHA plates were dried for 3–5 min followed by application of the disks on the surface of the agar. The diameter of inhibition zones was measured after 24 h incubation of the plates at 35ºC. *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923, and *S. aureus* ATCC 29213 was used as quality control strains.

PFGE

PFGE was performed to characterize MRSA and MSSA at genomic level as described previously, with a few modifications (McDougal *et al.*, 2003). Briefly, genomic DNA was prepared by mixing 200µl of standardized cell suspension in TE buffer (10 mmol/litre Tris–HCl and 1 mmol/litre EDTA, pH 8), 4µl of 1 mg/ml lysostaphin solution

(Sigma), and 200 µl of melted 1.5% Seakem Gold agarose. Sample plugs were then incubated in EC buffer (6 mmol/litre Tris-HCl, 1 mmol/litre NaCl, 100 mmol/litre EDTA, 0.5% Brij-58, 0.2% Sodium deoxycholate, and 0.5% Sodium lauroyl sarcosine) supplemented with lysozyme (Sigma) at final concentration of 1 mg/ml at 37°C for 4h, followed by overnight cell lysis at 54°C with 0.15 mg/ml of proteinase K (Sigma). Plugs were washed in TE buffer and then digested with 20U of *Sma I* (New England Biolab, Beverly, MA, USA) at 37^{o}C overnight. Electrophoresis was carried out on a CHEF-DR III apparatus (Bio-Rad) using the following parameters: initial switch time, 5 s; final switch time, 40 s; run time, 20 h; angle, 120; gradient, 6 V/cm; temperature, 14 C; ramping factor, linear. The PFGE patterns were analyzed using the BioNumerics software program (ver. 6.5; Applied Maths, Austin, TX, USA). Clustering was performed by using the Dice similarity coefficient and the unweighed pair group method with arithmetic means (UPGMA), with 1.5% of position tolerance and 1% optimization.

PCR-directed typing methods: SCC*mec* and *agr* typing were performed by PCR based approach. SCC*mec* types was designated by the combinations of *ccr* types and classes of *mec* regulatory genes. Cassette chromosome recombinase genes (*ccrA1*, *ccrB1*, *ccrA2*, *ccrB2*, *ccrA3*, *ccrB3*, and *ccrC*) and *mec* regulatory genes (*mecI*, *mecR1-A* for the membrane-spanning part of *mecR1*, and *mecR1-B* for the penicillin-binding part of *mecR1*) was be amplified by PCR. The contribution of *agr* to *S. aureus* virulence has been linked to its implication in gene regulation of virulence factors. The sequence of this hypervariable segment was target of PCR amplification for defining agr types (Novick, 2003). In addition, presence of the *pvl* gene was detected. PVL, a cytotoxin is associated

with increase virulence of *S. aureus* and is present in majority of CA-MRSA isolates (McClure *et al.*, 2006).

DNA sequence-based typing methods:

a. **MLST:** Seven housekeeping genes were amplified and sequenced for determination of allelic profiles. The seven genes include *arcC* for carbamate kinase, *aroE* for shikimate dehydrogenase, *glp* for glycerol kinase, *gmk* for guanylate kinase, *pta* for phosphate acetyltransferase, *tpi* for triosephosphate isomerase, and *yqiL* for acetyl coenzyme A acetyltransferase. For each locus, the sequences obtained from different bacteria were compared and the individual sequences are assigned allele numbers by utilizing the website for MLST Data Analysis (http://www.mlst.net/). For each isolate, seven alleles at each of the seven loci define its allelic profile which corresponds to its ST.

b. ***Spa* sequence typing:** *Spa* typing was performed in accordance with StaphyType standard protocol (http://spaserver.ridom.de). A *spa* type refers to the composition of the variable number of tandem repeats (VNTRs) in the 3' end of the spa gene. The *spa* gene was amplified, sequenced, and analyzed as described previously(Pu *et al.*, 2009).

MRSA isolates were characterized by Antimicrobial Susceptibility Tests, PFGE, MLST, SCC*mec* typing, and *spa* typing, whereas MSSA isolates were characterized by Antimicrobial Susceptibility Tests by Disc Diffusion, PFGE and MLST.

Results

Meat samples of beef, chicken, and turkey collected from 30 randomly-selected retail meat stores in Detroit, Michigan, from August 2009 to January 2010 was included in this study.

Sixty-five (22.5%) samples yielded *S. aureus*: 32 beef (20.5%), 19 chicken (25.0%), and 14 turkey (24.6%) samples. Six samples, consisting of 2 beef (1.3%), 3 chickens (3.9%), and 1 turkey (1.7%), were positive for MRSA as evidenced by the presence of *mec*(A).

Characterization of MRSA isolates:

Although an extra band was generated in MRSA 2a, 2b, 3, 5, and 6 by PFGE, all 9 MRSA isolates belonged to USA300. Multiple isolates from the same samples (MRSA 2a and 2b; MRSA 4a, 4b, and 4c) demonstrated indistinguishable PFGE patterns. Moreover, MLST, SCC*mec* typing, *agr* typing, and *pvl* detection showed all strains to be positive for ST8, SCC*mec* IVa, *agr* I, and Panton-Valentine leukocidin. However, *spa* typing identified 2 distinct *spa* types, t008 (11–19–12–**21–17**–34–24–34–22–25) and t2031 (11–19–12–**12–34–**34–24–34–22–25) (repeat variants in **boldface**), which differed by 5 nucleotides. t008, the most common *spa* type of USA300, was identified in 6 isolates of beef, chicken, and turkey origin, whereas t2031 was recovered from MRSA4a, 4b, and 4c from a chicken sample. The nucleotide variation in t2031 caused amino acid changes from glycine-asparagine in t008 to asparagine-lysine. The single nucleotide difference between repeats 12 (GGT) and 21 (GGC) and repeats 34 (AAA) and 17 (AAG) resulted in no amino acid change, with glycine and lysine encoded, respectively. The only multidrug-resistant MRSA isolate in this study (MRSA1) was from beef and was resistant to β-lactams, macrolides, and fluoroquinolones (Figure 4).

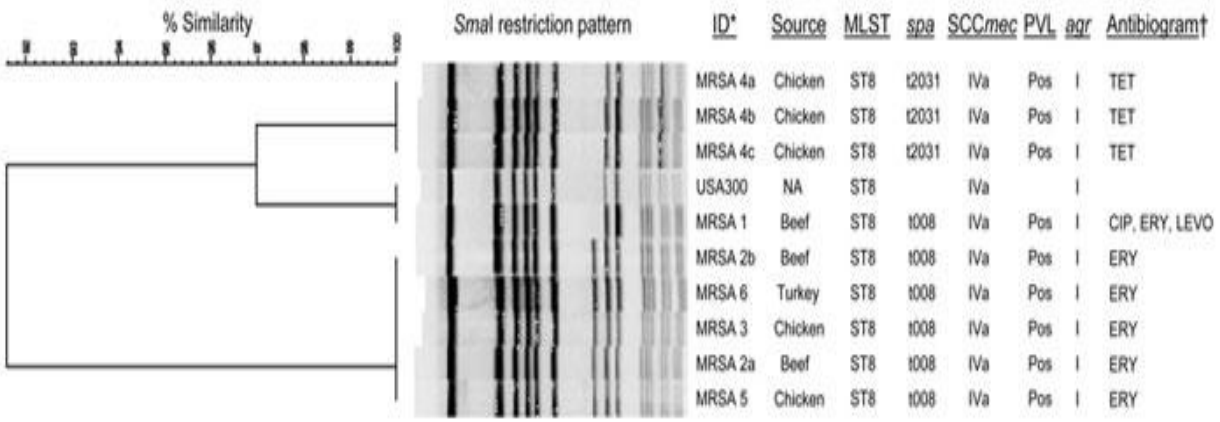
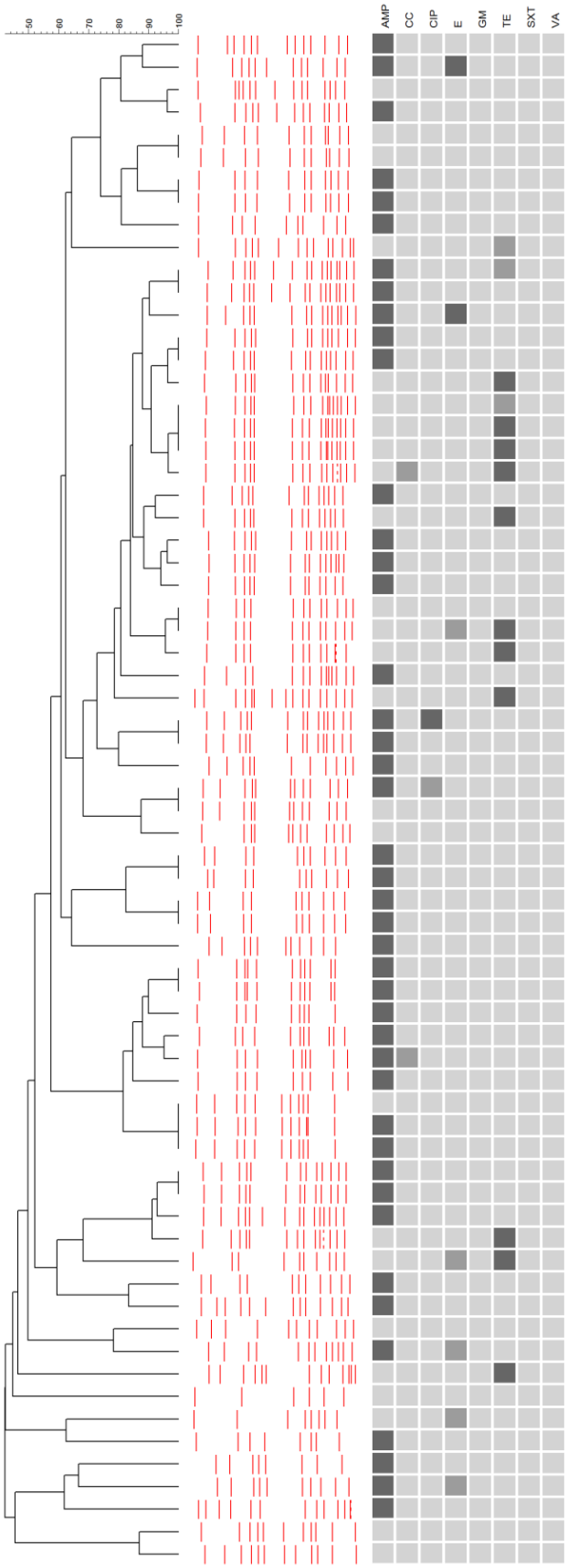


Figure 4. Dendrogram showing comparison of *Sma*I pulsed-field gel electrophoresis patterns, staphylococcal cassette chromosome (SCC) *mec* type, Panton-Valentine leukocidin (PVL) content, and *agr* type of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from meat samples. All MRSA isolates were resistant to β-lactam antimicrobial drugs (ampicillin, penicillin, and oxacillin) and grew on the 6 μg/mL of cefoxitin for screening methicillin resistance. *Isolates with the same arabic numbers were from the same sample; †only resistance to non–β-lactam antimicrobial drugs was listed. ID, identification; MLST, multilocus sequence typing; ST, sequence type; pos, positive; TET, tetracycline; NA, not available; CIP, ciprofloxacin; ERY, erythromycin; LEVO, levofloxacin.

Characterization of MSSA isolates:

A total of 71 MSSA isolates were examined by antimicrobial susceptibility testing against eight antimicrobials, including those used to treat staphylococcal infections. Although most isolates were resistant to ampicillin (61.9%) and tetracycline (21.1%), resistance phenotypes were not detected for four antimicrobials including gentamicin, clindamycin and trimethoprim/sulfamethoxazole and vancomycin. Ciprofloxacin and erythromycin resistance was identified in one isolate each. Of 57 antimicrobial resistant MSSA isolates, 51 were resistant to only one antimicrobial. Six isolates demonstrated resistance to two antimicrobials. They were one beef isolate resistant to ampicillin and ciprofloxacin, one chicken isolate resistant to ampicillin and erythromycin, and four turkey isolate resistant to ampicillin and tetracycline. No multidrug-resistant isolates were recovered. Intermediate resistance to ciprofloxacin, clindamycin, tetracycline and erythromycin was also observed (Figure. 5).

PFGE identified 52 unique patterns among the 69 typeable MSSA isolates. Overall similarity was 48% and *Sma I* digestion produced 9 to 15 fragments. Although most isolates from the same meat samples were indistinguishable, unique clones were also observed such as 38a and 38b. Meanwhile, identical PFGE patterns were identified from different meat samples, such as 28, 29 and 59 from beef samples. Sample 28 and 29 was collected on single day, however, 59 was collected after 3 weeks from same store (Figure. 5). Similar PFGE patterns were also found in strains isolated from different meat categories such as chicken-56 and turkey-19, beef-3 and chicken 57. It is important to note that these samples belonged to same store. Moreover, indistinguishable clone, such as 33a, 33b and 27a, 27b showed different antimicrobial susceptibility profiles.



ID	Source	Store ID	MLST	*pvl*
6	Beef	A	ST8	+
MRSA-2a	Beef	B	ST8	+
12	Beef	E	ST8	+
13	Beef	E	ST8	+
43	Chicken	M	ST8	
58	Chicken	M	ST8	
42a	Turkey	L	N	
42b	Turkey	L	N	
42c	Turkey	L	ST8	
54	Beef	E	ST8	+
22	Chicken	F	ST1	+
30	Chicken	F	ST1	
24	chicken	B	ST1	
3	Beef	B	ST1	
57	Chicken	B	ST1	
36	Chicken	G	ST1159	
59	Beef	A	ST1159	
29	Beef	A	ST1159	
28	Beef	A	ST1159	
46	Chicken	O	ST1159	
4	Turkey	C	ST1	
45	Turkey	O	ST1159	
9	Beef	B	ST1	
10	Beef	B	ST1	
8	Beef	B	ST1	
44	Beef	O	ST1159	
34	Beef	G	ST1159	
35	Turkey	G	ST1159	
38a	Beef	H	ST7	
53	Turkey	G	ST1159	
33b	Beef	G	ST1	
33a	Beef	G	ST1	
32	Beef	F	ST1	
27a	Beef	B	ST5	
27b	Beef	B	ST5	
26	Chicken	E	ST1	
56	Chicken	A	ST1767	
19	Turkey	A	ST1767	
1	Chicken	A	ST97	
2	Turkey	A	ST1767	
18	Chicken	A	ST1767	
31a	Beef	E	ST72	
31b	Beef	E	ST72	
21	Beef	S	ST72	
39	Turkey	J	ST72	
51	Turkey	J	ST1507	
52	Turkey	J	ST72	
7	Beef	A	ST8	+
23b	Beef	A	ST72	+
23a	Beef	A	ST72	+
50c	Chicken	Q	ST1004	
50b	Chicken	Q	ST1004	
50a	Chicken	Q	ST1004	
16	Beef	G	ST1159	
48	Chicken	P	ST1159	
47	Beef	Q	ST1767	
25	Chicken	C	ST14	
5	Beef	B	ST1635	
20	Beef	S	ST1159	
49	Beef	P	ST1159	
40	Beef	K	ST221	
55	Beef	K	ST461	
41	Turkey	L	ST8	
15a	Chicken	F	ST9	
15b	Chicken	F	ST9	
38b	Beef	H	ST8	
11	Beef	E	ST8	+
17	Beef	E	ST8	

Figure 5. PFGE dendrogram representing the genetic relatedness of 67 SmaI typeable MSSA strains and their antimicrobial susceptibility profiles. The four columns represents MSSA isolate code, source, store I.D., MLST type and presence of *pvl* gene, respectively.

Sixteen MLST sequence types were identified among the MSSA isolates, however, ST1, ST8 and ST1159 dominated the collection (Figure. 7). Prevalence of ST8 and ST1159 in all meat categories ranged between 11-23% and 17-23% respectively. ST1 was mostly recovered in chicken (26%) and beef (20%) when compared with turkey (6%). Two indistinguishable MSSA strains from turkey was assigned to a novel single locus variant of MLST 1159 with allelic profile, 3-4-4-1-4-4-3. Four ST398 strains were found in two samples of turkey that were collected three weeks apart from the same store. All of them were resistant to ampicillin and tetracycline (Figure. 6). Of particular note, all tetracycline resistant isolates were found to be ST1159 and were susceptible to ampicillin.

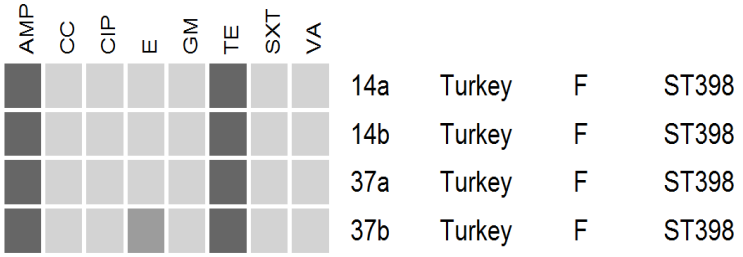


Figure 6. Antimicrobial susceptibility profiles of four ST398 strains recovered in this study.

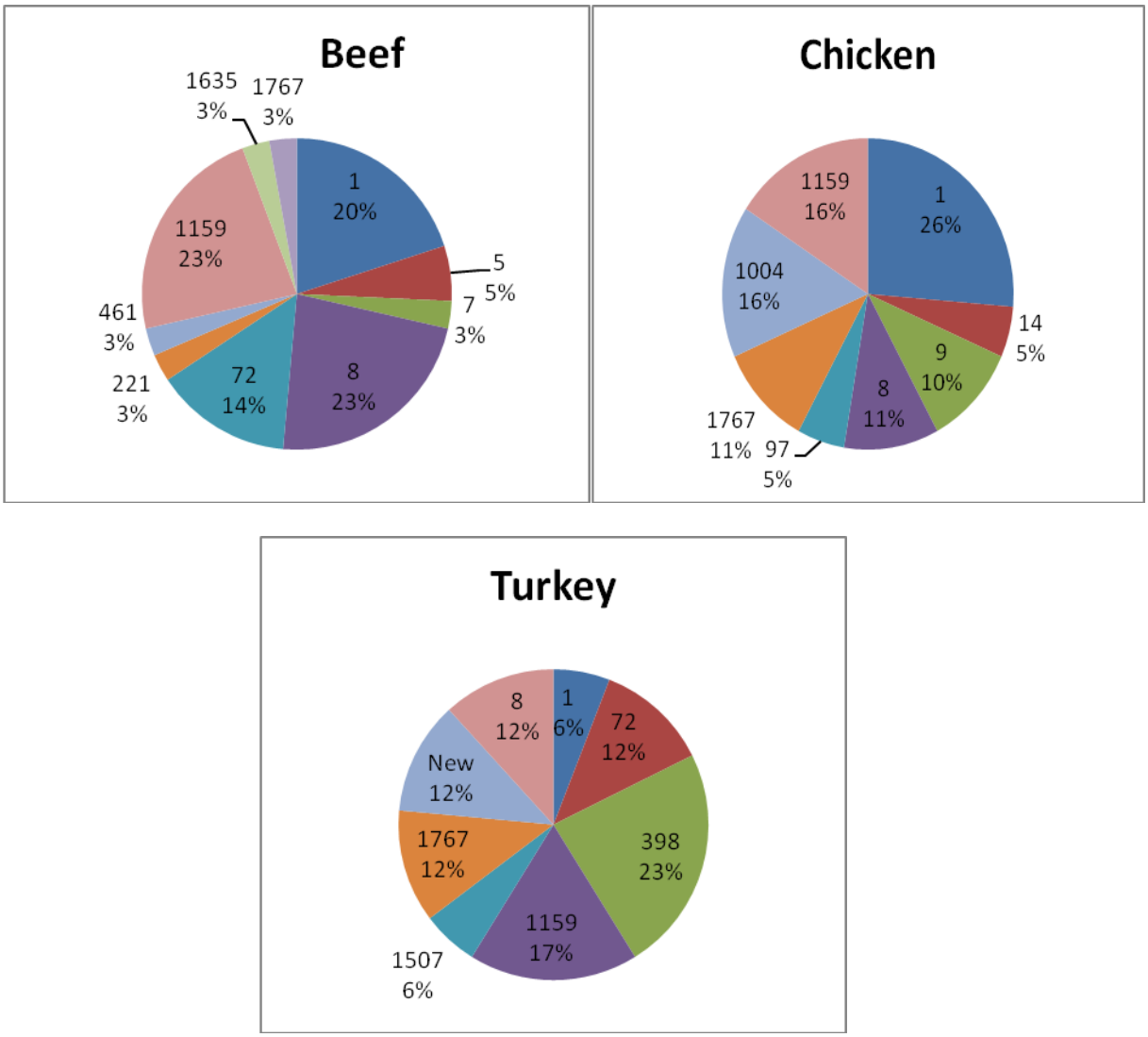


Figure 7. Distribution of Sequence Types (STs) of MSSA isolated from beef, chicken and turkey

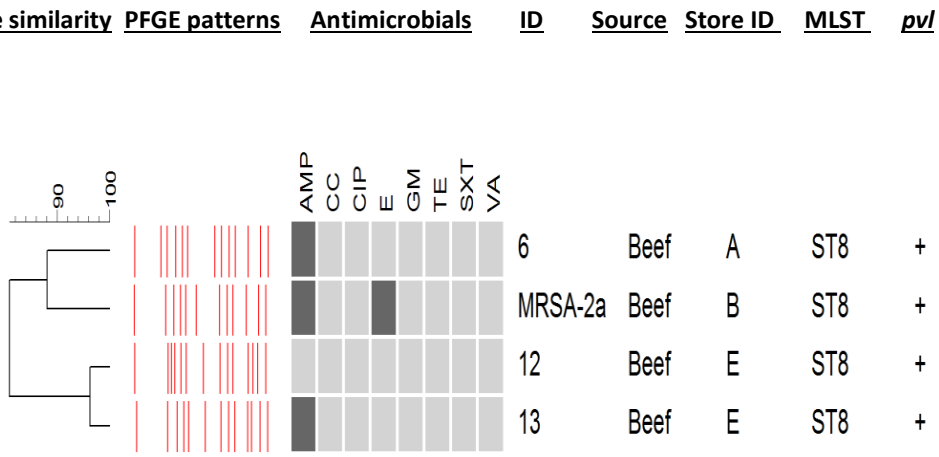


Figure 8.

PFGE dendrogram representing the genetic relatedness of three MSSA strains with USA300 and their antimicrobial susceptibility profiles. The four columns represents MSSA isolate code, source, store I.D., MLST type and presence of *pvl* gene, respectively.

As shown in Figure 8, three isolates with more than 80% similarity to PFGE profile of USA300 strain are recovered in this study. All the three isolates were from three unique samples of beef (6, 12 and 13) where sample 12 and 13 belonged to same store. Moreover, MLST and *pvl* detection showed all strains to be positive for ST8 and Panton-Valentine leukocidin. These isolates were sensitive to all the classes of antimicrobials tested except two strains showing resistance to ampicillin. Overall, prevalence of *pvl* gene in MSSA isolates was 12.6% and the gene was predominantly recovered from strains of beef origin. Nine strains positive for *pvl* gene included six ST8 and two ST72 strains from beef samples, and one ST1 strain from chicken sample.

Discussion

The present study demonstrated retail meat as a reservoir of *S. aureus* including, MRSA and MSSA. The overall lower prevalence of *S. aureus* and MRSA than that found in a previous study in the United States (40% and 5%, respectively) (Pu *et al.*, 2009) might be explained by our exclusion of pork because pork and swine production have been major reservoirs of MRSA (De Boer *et al.*, 2009; Smith and Pearson, 2011). However, different geographic location and cold sampling seasons in this study also might have caused the variations. Unlike studies in Europe, where researchers have reported the animal MRSA clone ST398 from various meat products (De Boer *et al.*, 2009), all MRSA isolates in our study were USA300, which suggests a possible human source of contamination during meat processing (Pu *et al.*, 2009). The failure to identify ST398 in the US retail meat also indicates that the human MRSA clones might be better adapted in meat processing than ST398 in this country. Since ST398 is widespread in animals and meat in Europe and has been isolated from other parts of the world (Weese *et al.*, 2010), it is not too bold to predict that ST398 might appear in US meat in the future, especially after the recent report of ST398 from US swine (Smith and Pearson, 2011).

The 5-nt difference between t2031 and t008 implicates multiple MRSA clones in poultry. Previous studies have shown *spa* variants of USA300 from clinical cases associated with distinctive symptoms (Larsen *et al.*, 2009; Shibuya *et al.*, 2008). A single repeat variant, t024, showed substantial genetic, epidemiologic, and clinical differences from t008 in Denmark (Larsen *et al.*, 2009). Researchers in Japan also recovered 2 *spa* variants of USA300: t024, which causes blood infections, and t711, which is associated with subcutaneous abscesses (Shibuya *et al.*, 2008). In both studies, t024 behaved as hospital-associated MRSA, suggesting that *spa* variants of USA300 could lead to different clinical outcomes. Therefore, we can reasonably

assume that variants with a meat origin also might have different public health implications. Despite the recovery of MRSA from retail chicken and t2031 that has an antibiogram distinct from t008, except for β-lactam resistance, several questions remain about whether more *spa* variants are present in poultry (or meat). These include whether t2031 is more adaptable to chicken production because of the 2 amino acid differences from t008, or whether t2031 is linked with specific antimicrobial drug resistance phenotypes other than β-lactam resistance.

The MSSA PFGE data suggest an overall diverse population. The identification of indistinguishable clones in different meat samples and categories from the same store suggests clonal persistence of *S. aureus* and linkage of hygienic conditions of stores with contamination of *S. aureus*. *S. aureus* contamination of carcass by meat handlers is quite common and has been demonstrated using PFGE as an epidemiological tool in a previous study (Vanderlinde *et al.*, 1999).

Dominance of ST1 and ST1159 was consistent with a previous US multistate study that has also reported high prevalence of ST1 and ST1159 in beef and pork (Waters *et al.*, 2011). However, we found large proportion of these ST types in all meat categories compared to the previous study where ST1 and ST1159 was only observed in pork and beef, respectively. Recovery of ST1, a human lineage and common sequence type of CA-MRSA in all meat categories indicates human contamination (Diep *et al.*, 2006). Likewise, ST1159 is a human colonizer and has been isolated from healthy adults (Sakwinska *et al.*, 2009). ST1004 and ST14 have been found in MRSA from humans in malaysia and spain respectively (Shamsudin *et al.*, 2008; Argudín *et al.*, 2011). Our collection also comprised of high level of ST8 MSSA strains which is a common ST type of CA-MRSA in US (Mediavilla *et al.*, 2012). We identifed ST5 and ST72, one of the most globally disseminated hospital-associated MRSA (HA-MRSA) lineages in

our study (Takano *et al.*, 2013; Joo *et al.*, 2012). It was not surprising to find ST5 and ST72 in MSSA, since these lineages have been recovered in MRSA strains from meat (Lim *et al.*, 2010; Ko *et al.*, 2011; Jackson *et al.*, 2013). All of these findings strongly indicate common reservoir of *S. aureus* shared between human and agricultural products.

Livestock related lineage ST398, observed in four isolates from turkey in our study was also reported as highly prevalent in turkey (79%) previously (Waters *et al.*, 2011). Therfore, we may assume that turkey is a major reservoir of this lineage when compared to other meat categories. We also observed porcine relate lineage, ST9, in chicken isolates. MRSA ST9 have been isolated from chicken meat in Germany (Feßler *et al.*, 2011), turkey meat in Iowa (Hanson *et al.*, 2011) and recently from retail pork in Georgia (Jackson *et al.*, 2013). Further, the identification of single locus variant of ST1159 supports the idea that meat may act as a reservoir for evolution of existing clones and emergence of new MSSA lineages.

The high prevalence of resistance to ampicillin and tetracycline was not surprising as compared to previous studies on antimicrobial resistance profile of *S. aureus* from meat and poultry in the US and Italy (Waters *et al.*, 2011; Pesavento *et al.*, 2007). When comparing these three studies, ampicillin and tetracycline resistance predominated in all the studies ranging between 42-60% and 19-62%. On the other hand, erythromycin and clindamycin resistance was not observed in our study when compared to others, where it ranges between 16-19% and 10-21%. Overall, multi-resistance observed in this study was much lower than reported in other multistate US study, where 56% isolates showed resistance to two or more antimicrobials. Different sampling locations and antimicrobial susceptibility testing procedures may be some of the factors accounting for differences observed. Most of these antimicrobials including ampicillin and tetracycline antimicrobials are approved for US food animal production, so this

may be indication of extensive use of these antimicrobials in meat production (Marshall and Levy, 2011; Silbergeld *et al.*, 2008).

In our study MLST shows good correlation with antimicrobial susceptibility data but less discriminatory power than PFGE. High discriminatory power of PFGE for *S. aureus* has been demonstrated in previous studies (Peacock *et al.*, 2002) and application of multiple sub typing methods is highly recommended. Interestingly, all tetracycline resistant isolates were ST1159 indicating strong linkage between this lineage and tetracyline resistance.

Recovery of three unique isolates from beef with more than 80% similarity to PFGE profile of USA300 strain indicates the possibility of emergence of MRSA from these MSSA strains. Further, presence of PVL gene in these isolates strengthen our hypothesis as PVL gene is commonly associated with MRSA clone USA300 (Rybak and LaPlante, 2005). Clonal relatedness of community-acquired MSSA and MRSA have been described in a study investigating genetic relatedness of five CA-*S. aureus* isolates (Mongkolrattanothai *et al.*, 2003). In this study, researchers concluded that CA-MRSA infection arose from MSSA isolates that successfully incorporated the SCC*mec* IV element. In addition, MLST results identified these isolates as ST8 which is again common ST type of USA300 in US (Mediavilla *et al.*, 2012). Apart from USA 300 background, MSSA collection also contained isolates exhibiting other MLST types recovered in MRSA. MRSA ST8, ST5, ST9 and ST72 has been reported in meat previously (Pu *et al.*, 2009; De Jonge *et al.*, 2010; Lim *et al.*, 2010). So, we can assume that these MSSA strains clearly represent a genetic background favorable for the emergence of MRSA.

In conclusion, *S. aureus* was recovered from 22.4 % of meat samples including 6 samples carrying MRSA, suggesting meat products as important reservoir of these commensals. Nine

MRSA isolates recovered in this study were resistant to atleast one non β-lactam antimicrobial, including one isolate which was multidrug resistant indicating that non β-lactam resistance is also prevalent in MRSA of food origin. All MRSA isolates were human clone, suggesting a possible human contamination. They all exhibited similar molecular profiles (SCC*mec* IV, ST8, *pvl* positive and *agr*I) by various subtyping methods, except for *spa* typing, which identified 2 distinct *spa* types, t008 and t2031. Recovery of t2031, a *spa* variant of USA300, from a chicken product raises questions on its emergence, antimicrobial resistance and virulence potential. Further, prevalence of common MRSA ST types ST8, ST5, ST9 and ST72 identified in MSSA in our study indicates the possibility of emergence of MRSA from these MSSA strains. Whether CA-MRSA emerged in farms, community or retail meat products is unknown, however, risk of colonization to consumers and person handling the meat with MRSA exist.

CHAPTER 3

METHICILLIN-RESISTANT COAGULASE-NEGATIVE STAPHYLOCOCCI IN US RETAIL MEAT

Introduction

Coagulase-negative staphylococci (CoNS) are commonly found in food, environment, and human clinical settings (Bagcigil *et al.*, 2007; Kawano *et al.*, 1996; Huber *et al.*, 2011; Zong *et al.*, 2011). Methicillin-resistant CoNS (MRCoNS) have been suggested as a source of *mec*(A), the methicillin resistance gene, which has the potential to transfer to *Staphylococcus aureus* (*S. aureus*), a species that shows the highest pathogenic potential among staphylococci (Ito *et al.*, 2009). Two lines of evidence have supported this hypothesis. 1) CoNS are a larger reservoir of *mec*(A) than *S. aureus* (Martins and Cunha, 2007; Diekema *et al.*, 2001) and may contribute to the emergence of MRSA. Recent findings of 99-100% sequence similarity of a *mec*(A) homologue present in *S. fleuretti*, an animal-related staphylococcal species and a member of *S. sciuri* group, indicate that the direct precursor of *mec*(A) in MRSA may be present in this group of CoNS (Tsubakishita *et al.*, 2010). 2) Staphylococcal cassette chromosome *mec* (SCC*mec*) types are more heterogeneous in CoNS than those in MRSA (Tulinski *et al.*, 2012). SCC*mec* is a mobile genetic element inserted into the staphylococcal chromosome that carries *mec*(A). The origin of SCC*mec* is unknown, but it is speculated that methicillin resistance genes evolved in CoNS and then horizontally transferred between staphylococcal species (Hanssen and Ericson Sollid, 2006). Research examining *S. aureus* and CoNS from pig farms identified common SCC*mec* types shared in *S. aureus* and *S. epidermidis* from the same environmental niche, indicating the possibility of interspecies transfer of SCC*mec* (Tulinski *et al.*, 2012).

The *S. sciuri* group has been speculated as the origin of *mec*(A) (Tsubakishita *et al.*,

2010). It includes 4 species: *S. sciuri*, *S. vitulinus*, *S. lentus*, and *S. fleurettii*. These species are usually isolated from animals and food of animal origin, but not commonly found in humans (Kawano *et al.*, 1996; Feßler *et al.*, 2010; Khadri and Alzohairy, 2010; Bagcigil *et al.*, 2007). Meat and animal products are expected to be a significant reservoir of MRCoNS. A recent study found multiple species of MRCoNS to be prevalent in bulk tank milk and minced meat in Europe and that *S. sciuri* and *S. fleuretti* were predominant (Huber *et al.*, 2011), raising concerns on their role in *mec*(A) transfer. Unfortunately, no SCC*mec* data were provided in that study. Since MRSA has been isolated in US retail meat (Bhargava *et al.*, 2011; Pu *et al.*, 2009), questions arise as to how MRCoNS in retail meat may contribute to the emergence of MRSA in this country.

SCC*mec* types are defined by the combination of *ccr* and *mec* gene complex (Kondo *et al.*, 2007). *ccr* is the cassette chromosome recombinase gene complex and responsible for the mobility of the element and its surrounding sequences. The *mec* gene complex includes *mec*(A) and its regulatory genes and insertion sequences, such as IS*431*. Some of the SCC*mec* types have been associated with multi-drug resistance as these cassettes contain antimicrobial resistance genes on integrated plasmids (pUB110, pT181) or a transposon (Tn*554*) (Hanssen and Ericson Sollid, 2006). Of 11 SCC*mec* types (I through XI) identified in *S. aureus* so far (Shore *et al.*, 2011), SCC*mec* types II, III, IV, and V have been recovered from MRSA of meat and animal origin (Bhargava *et al.*, 2011; Pu *et al.*, 2009; Feßler *et al.*, 2010; Cui *et al.*, 2009; Nemati *et al.*, 2008; Lozano *et al.*, 2009; Lim *et al.*, 2010). Because most SCC*mec* typing schemes have been based on MRSA, many SCC*mec* types are undetermined in CoNS (Hanssen and Ericson Sollid, 2006; Zhang *et al.*, 2009; Descloux *et al.*, 2008), especially those of environmental origin. SCC*mec* types I, II, III, IV, V, VI and VIII have been identified in MRCoNS (Zong *et al.*, 2011;

Feßler *et al.*, 2010; Tulinski *et al.*, 2012; Zhang *et al.*, 2009; Vanderhaeghen *et al.*, 2012), of which I, III, IV, V, VI were reported from cattle, pig farms and bovine mastitis while no data are available on SCC*mec* types of MRCoNS isolated from meat.

This study was aimed to understand the SCC*mec* composition in CoNS of different genetic background from US retail meat. The data will provide valuable insight into the extent of MRCoNS serving as the *mec*(A) reservoir.

Materials and Methods

CoNS isolates

Isolation of CoNS was performed on 289 raw meat samples (156 beef, 76 chicken, and 57 turkey) collected from a previous study (Bhargava *et al.*, 2011). Isolates were identified by Gram-staining, coagulase test and *Staphylococcus* genus specific PCR (Morot-Bizot *et al.*, 2004).

Determination of methicillin resistance and staphylococcal species identification

Phenotypic cefoxitin resistance of CoNS was determined by broth dilution test (Zhang *et al.*, 2011). Minimum Inhibitory Concentration (MIC) of cefoxitin was determined using an inoculum of 5×10^5 CFU/ml of the bacterial culture on Mueller-Hinton Broth (CAMHB, Difco, Detroit, MI, USA) supplemented with 2% of NaCl as well as cefoxitin (Sigma, St Louis, MO, USA) in concentrations ranging from 1 to 64 µg/ml. The resistance breakpoint used was 8 µg/ml. All confirmed CoNS isolates were examined by PCR for the presence of *mec*(A) (Vannuffel *et al.*, 1995). A sample was considered positive for MRCoNS if at least one isolate from that sample was positive for *mec*(A). The CoNS species was identified by sequencing the 429 bp PCR amplicon of the superoxide dismutase (*sodA*) gene as described previously (Poyart *et al.*,

2001) and compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) program.

SCC*mec* typing and amplification of IS*431* and Tn*554*

DNA template was prepared by the Genomic DNA Isolation Kit (Promega, Madison, WI, USA). PCR was performed to amplify cassette chromosome recombinase genes (*ccrA1*, *ccrB1*, *ccrA2*, *ccrB2*, *ccrA3*, *ccrB3*, and *ccrC*) and *mec* regulatory genes (*mecI*, *mecR1A* for the membrane-spanning part of *mecR1*, and *mecR1B* for the penicillin-binding part of *mecR1*) (Hanssen *et al.*, 2004; Deurenberg *et al.*, 2005; Vannuffel *et al.*, 1995). The combinations of *ccr* types and classes of *mec* gene complexes were used to differentiate the SCC*mec* types among isolates. For the isolates untypeable by this method, a multiplex PCR was performed targeting the *ccr* gene complex (Kondo *et al.*, 2007). Moreover, presence of IS*431* and Tn*554* was also examined by target-specific PCR (Zhang *et al.*, 2009). When the combination of *ccr* types, *mec* gene complex, and Tn*554* failed to designate the SCC*mec* types, then SCC*mec* types were determined solely on the *ccr* types (Deurenberg *et al.*, 2005; Zhang *et al.*, 2009). Strains were classified as untypeable when none of the *ccr* genes was amplified.

Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out to characterize MRCoNS at genomic level as described previously, with a few modifications (Zhang *et al.*, 2009; McDougal *et al.*, 2003). Briefly, genomic DNA was prepared by mixing 200µl of standardized cell suspension in TE buffer (10 mmol/L Tris–HCl and 1 mmol/L EDTA, pH 8), 4 µl of 1 mg/ml lysostaphin solution (Sigma, St Louis, MO, USA), and 200 µl of melted 1.5% Certified Megabase agarose (Bio-Rad Laboratories, CA, USA). Sample plugs were then incubated in EC buffer (6 mmol/L Tris-HCl, 1 mmol/L NaCl,

100 mmol/L EDTA, 0.5% Brij-58, 0.2% Sodium deoxycholate, and 0.5% Sodium lauroyl sarcosine) supplemented with lysozyme (Sigma, St Louis, MO, USA) at the final concentration of 1 mg/ml at 37°C for 4 h, followed by overnight cell lysis at 54°C with 0.15 mg/ml of proteinase K (Sigma, St Louis, MO, USA). Plugs were washed in TE buffer and then digested with 20 U of *Sma* I (New England Biolab, Beverly, MA, USA) at 37^{o}C overnight. Electrophoresis was carried out on a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA, USA) using the following parameters: initial switch time, 5 s; final switch time, 40 s; run time, 20 h; angle, 120°C; gradient, 6 V/cm; temperature, 14°C; ramping factor, linear. The PFGE patterns were analyzed using the BioNumerics software program (ver. 6.5; Applied Maths, Austin, TX, USA). Clustering was performed by using the Dice similarity coefficient and the unweighed pair group method with arithmetic means (UPGMA), with 1.5% of position tolerance and 1% optimization. *Staphyloccus aureus* NCTC8325 was utilized as reference standard.

RESULTS

Diversity of MRCoNS in meat

MRCoNS were isolated from 25 samples, consisting of 17 beef, 5 chickens, and 3 turkey samples. One to three isolates were selected from each sample for further characterization. Among 51 isolates recovered, seven CoNS species were identified, including 28 *S. sciuri*, 9 *S. fleurettii*, 5 *S. epidermidis*, 5 *S. lentus*, 2 *S. vitulinus*, 1 *S. pasteuri* and 1 *S. saprophyticus* (Table 5). There was no clear distinction among meat categories regarding the MRCoNS distribution. Beef carried all species but *S. vitulinus*. Chicken and turkey were contaminated by only 3 species each. *S. sciuri* was the only species that was recovered from all meat categories. *S. sciuri* was the predominant species from beef (10 of 16 samples), whereas *S. fleuretti* and *S. sciuri* were recovered from 3 and 2 out of 6 chicken samples, respectively. Although all 51 MRCoNS

isolates harbored the *mec*(A) gene, phenotypic resistance to cefoxitin was not observed in five isolates consisting of *S. vitulinus* (2), *S. pasteuri* (1), *S. sciuri* (1), and *S. saprophyticus* (1). The majority of *S. sciuri* (24 of 28 isolates) showed high MIC (64 µg/ml) whereas *S. fleuretti*, *S. epidermidis* and *S. lentus* exhibited MIC values of 8 or 16 µg/ml (Figure 9).

Table 5. Distribution of MRCoNS species in meat samples

Meat type	No. of MRCoNS+ samples (No. of isolates)						
	S. sciuri	*S. fleuretti*	*S. epidermidis*	*S. lentus*	*S. vitulinus*	*S. pasteuri*	*S. saprophyticus*
Beef	10(19)	1(2)	1(3)	2(4)	0	1(1)	1(1)
Chicken	2(6)	3(7)	0	0	1(2)	0	0
Turkey	1(3)	0	1(2)	1(1)	0	0	0
Total	13(28)	4(9)	2(5)	3(5)	1(2)	1(1)	1(1)

Heterogeneity of SCC*mec* types in MRCoNS

SCC*mec* types were determined in 32 MRCoNS isolates from 16 samples (Figure 9). Twenty-one isolates from 11 meat samples (6 beef, 2 chicken, and 3 turkey samples) were designated as SCC*mec* I, III, IV, V, and composite types by the first PCR method. Additional 11 isolates from 5 samples (4 beef and 1 chicken samples) were typed by the second method using multiplex PCR as SCC*mec* I, III, and V. SCC*mec* I was exhibited in all meat categories, whereas SCC*mec* V was identified in beef and chicken, and so were SCC*mec* III and IV in beef and turkey only, respectively. Most SCC*mec*-typeable isolates belonged to *S. sciuri*, *S. fleuretti*, and *S. lentus*. *S. sciuri* and *S. fleuretti* had the most diverse SCC*mec* types. SCC*mec* I, III, V, and untypeable were identified in *S. sciuri*, and *S. fleuretti* were consisted of I, V, and untypeable. SCC*mec* I was designated in 13 *S. sciuri*, 3 *S. fleuretti* and 1 *S. lentus*. Four *S. lentus* and 2 *S.*

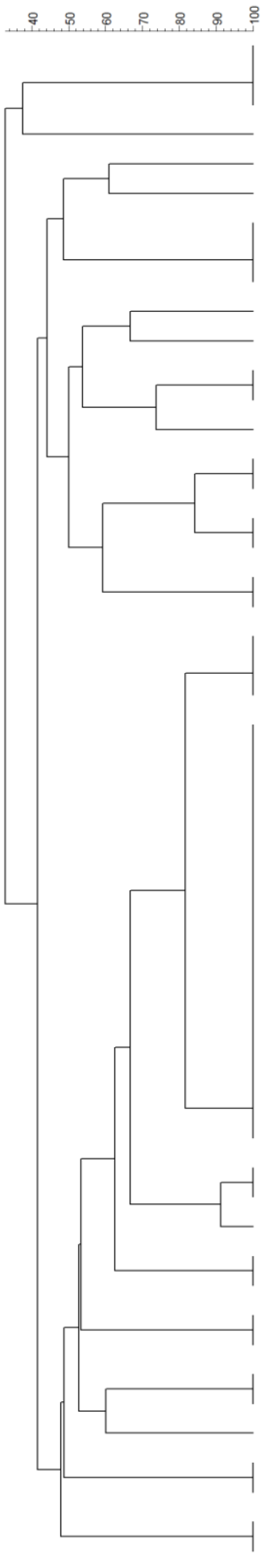
sciuri from beef samples showed SCC*mec* III. Two isolates of *S. epidermidis* from one turkey sample were typed as SCC*mec* IV. Four *S. fleuretti* from a beef and a chicken sample and one *S. scuiri* from beef were classified as SCC*mec* V. Single isolates of *S. pasteuri* and *S. saprophyticus* exhibited composite I+V and III+V elements, respectively. IS*431* was not detected in 10 isolates, including 7 belonging to SCC*mec* I and V. Tn*554* was identified in 7 isolates including 3 SCC*mec* I and 4 type III.

SCC*mec* typing failed to determine the SCC*mec* identity in 19 isolates, but a large variety of *mec* regulatory complex was observed in these isolates. The 19 isolates were consisted of 12 *S. sciuri*, 3 *S. epidermidis*, 2 *S. fleuretti*, and 2 *S. vitulinus*. Twelve of them carried all the *mec* regulatory genes (*mecI*, *mecR1A*, and *mecR1B*). They were 7 *S. sciuri* and 3 *S. epidermidis* from beef, and 2 *S. fleuretti* from chicken. The remaining 5 *S. sciuri* and 2 *S. vitulinus* carried *mecR1A* only, *mecI* and *mecR1A*, *mecI* and *mecR1B*, and none of the genes (2 *S. vitulinus*). IS*431* was also detected in 16 untypeable isolates except for 3 *S. sciuri* from chicken.

Diversity of MRCoNS

A total of 21 PFGE patterns were observed (Figure 9). Isolates from same samples showed indistinguishable PFGE patterns. Overall, clonal relatedness was mainly observed in SCC*mec* I and III isolates, such as samples 2, 15, 16, and 17 for SCC*mec* I and samples 23 and 24 for SCC*mec* III. Isolates from other SCC*mec* types showed distant relationships at the genomic level. Indistinguishable *S. sciuri* clones were observed in 5 different meat samples belonging to 3 meat types. PFGE was also able to group 18 of 28 *S. sciuri* isolates in the same cluster with more than 80% similarity and 12 of these isolates were typed as SCC*mec* I. Four *S. lentus* determined as SCC*mec* III recovered from beef were clustered at 85% similarity, though distinct from the remaining one *S. lentus* typed as SCC*mec* I from turkey. *S. fleuretti* from 4

different beef and chicken samples were clonally distinct, and so were *S. epidermidis* from beef and turkey.



19a	S. fleuretti	Chicken	8	I	+	
19b	S. fleuretti	Chicken	8	I	+	
19c	S. fleuretti	Chicken	8	I	+	
21	S. saprophyticus	Beef	2	III+V	+	
5	S. lentus	Turkey	16	I	+	
6	S. sciuri	Beef	64	I	+	
13a	S. epidermidis	Beef	16		+	
13b	S. epidermidis	Beef	16		+	
13c	S. epidermidis	Beef	16		+	
12	S. sciuri	Beef	64		+	
NCTC8325						
22a	S. fleuretti	Chicken	16	V	+	
22b	S. fleuretti	Chicken	16	V	+	
4	S. sciuri	Beef	4	V		
24a	S. lentus	Beef	16	III	+	
24b	S. lentus	Beef	16	III	+	
23a	S. lentus	Beef	16	III	+	+
23b	S. lentus	Beef	16	III	+	+
8a	S. fleuretti	Chicken	16		+	
8b	S. fleuretti	Chicken	16		+	
2a	S. sciuri	Beef	8	I		+
2b	S. sciuri	Beef	8	I		+
2c	S. sciuri	Beef	8	I		+
14a	S. sciuri	Beef	64		+	
14b	S. sciuri	Beef	64		+	
14c	S. sciuri	Beef	64		+	
15a	S. sciuri	Turkey	64	I	+	
15b	S. sciuri	Turkey	64	I	+	
15c	S. sciuri	Turkey	64	I	+	
16a	S. sciuri	Chicken	64	I	+	
16b	S. sciuri	Chicken	64	I	+	
16c	S. sciuri	Chicken	64	I	+	
17a	S. sciuri	Beef	64	I		
17b	S. sciuri	Beef	64	I		
17c	S. sciuri	Beef	64	I		
1a	S. sciuri	Chicken	64			
1b	S. sciuri	Chicken	64			
1c	S. sciuri	Chicken	64			
3a	S. sciuri	Beef	64		+	
3b	S. sciuri	Beef	64		+	
11	S. sciuri	Beef	64		+	
20a	S. sciuri	Beef	64	III	+	+
20b	S. sciuri	Beef	64	III	+	+
9a	S. sciuri	Beef	64		+	
9b	S. sciuri	Beef	64		+	
18a	S. fleuretti	Beef	16	V	+	
18b	S. fleuretti	Beef	16	V	+	
10	S. pasteuri	Beef	2	I+V	+	
7a	S. epidermidis	Turkey	16	IV	+	
7b	S. epidermidis	Turkey	16	IV	+	
25a	S. vitulinus	Chicken	2		+	
25b	S. vitulinus	Chicken	2		+	

Figure 9. PFGE dendrogram representing the genetic relatedness and SCC*mec* compositions of 51 MRCoNS

*, Isolates with the same Arabic numbers were from the same sample.

Discussion

MRCoNS are an important reservoir of *mec*(A) and have been speculated as the precursor of MRSA. Recovery of diverse population of MRCoNS comprising of heterogeneous and composite SCC*mec* types in our study suggests meat as an important environmental source of MRCoNS. The study also shows the clonal distribution of MRCoNS, such as *S. sciuri*, in different meat categories and horizontal transmission of same SCC*mec* types among Staphylococcal species. The ancestral species of *mec*(A), such as *S. sciuri* and *S. fleueretti*, carried multiple SCC*mec* types, indicating great potential of these species to contribute to the emergence of MRSA in the environment.

The role of CoNS, especially those species in the *S. sciuri* group, in the *mec*(A) evolution has been studied recently. Three *mec*(A) gene homologues have been found in *S. sciuri*, *S. vitulinus*, and *S. fleurettii*, with 80%, 91%, and 99-100% homology, respectively, with *mec*(A) in MRSA (Couto *et al.*, 1996; Schnellmann *et al.*, 2006; Tsubakishita *et al.*, 2010). The *mec*(A) complex in *S. fleurettii* also matches that in the MRSA strain N315 (Tsubakishita *et al.*, 2010), indicating strongly the importance of MRCoNS in MRSA evolution. The predominance of *S. sciuri* and *S. fleuretti* in the 7 Staphylococcal species identified in this study is in consistency with a Swiss study (Huber *et al.*, 2011), though *S. fleuretti* was more prevalent than *S. sciuri* in that study. In comparison with the high prevalence of *S. sciuri* in beef, *S. fleuretti* outnumbered *S. sciuri* in chicken, suggesting the possible variation of CoNS species in host adaptation. The source of CoNS contamination in meat can be from animals and human, as evidenced in this

study by the recovery of common animal colonizers, such as *S. sciuri* and *S. fleuretti* (Thurlow *et al.*, 2012), and a human associated species, *S. epidermidis (Widerstrom et al., 2012)*. Interspecies variation exists in the extent of resistance reservoir in MRCoNS as evidenced by different resistance levels among species, especially between *S. sciuri* and *S. fleuretti*. The higher MIC observed in *S. sciuri* than that in *S. fleuretti* suggests different tendency to develop the resistance phenotype among species carrying *mec*(A). Regardless, *mec*(A) itself is still a risk due to its potential transfer across species.

SCC*mec* I and III are primarily associated with hospital acquired MRSA (Martins and Cunha, 2007) and have also been recovered from MRCoNS in various animals (Zhang *et al.*, 2009; Tulinski *et al.*, 2012), although SCC*mec* I is still rare in MRCoNS. Their dominance in our meat isolates (23 of 32 SCC*mec*-typeable CoNS) signifies the common pool of SCC*mec* elements in human, animals, and meat and possible genetic exchange among Staphylococcal species when present in the same environmental niche. This is also supported by the identification of SCC*mec* III, IV, and V, types previously found in MRSA of meat and animal origin (Bhargava *et al.*, 2011; Pu *et al.*, 2009; Feßler *et al.*, 2010; Cui *et al.*, 2009; Nemati *et al.*, 2008; Lozano *et al.*, 2009; Lim *et al.*, 2010), in MRCoNS in this study. Identification of heterogeneous SCC*mec* types in *S. sciuri* and *S. fleuretti* strengthens the hypothesis that MRCoNS are a potential source of *mec*(A) that may contribute to the emergence of MRSA. Considering the small number of *S. fleuretti* isolates (9), which carried SCC*mec* I, V, and untypeable, it is reasonable to assume that a much higher degree of diversity of SCC*mec* types may exist in this group of CoNS. The carriage of SCC*mec* IV by *S. epidermidis* is also of significance as high homology of SCC*mec* IVa between *S. epidermidis* and MRSA USA300 and 400 (Barbier *et al.*, 2010) and interspecies horizontal transfer of SCC*mec* from *S. epidermidis* to

S. aureus (Wielders *et al.*, 2001) have been identified. Again, MRCoNS in food and food production environment may serve as the *mec*(A) reservoir for MRSA. Multiple CoNS species sharing the same SCC*mec* types also indicate the potential of cross species transfer of SCC*mec* regardless of the genetic background of host strains.

Composite SCC*mec* elements have been reported in both MRSA and MRCoNS, although they are more common in MRCoNS (Zong *et al.*, 2011; Coombs *et al.*, 2011). The two composite SCC*mec* elements reported here were both from beef, exhibiting SCC*mec* III+V and I+V. The carriage of multiple *ccr* genes and absence of all or two (except for *mecR1A*) *mec* regulatory genes (data not shown) in these two isolates suggests complex gene recombination and rearrangement in the genomes of CoNS, which may generate novel types of SCC*mec* elements (Hanssen and Ericson Sollid, 2006). The susceptible phenotype of these two isolates to cefoxitin is interesting as this may imply the change of methicillin resistance due to the acquisition of composite SCC*mec* units. The prevalence of untypeable SCC*mec* in MRCoNS in this study is another indication of a larger reservoir of SCC*mec* in MRCoNS than MRSA.

The diverse genetic background of *S. sciuri* and *S. fleuretti* from all meat samples suggests the widespread distribution of these two species in meat and the significance of them serving as the *mec*(A) reservoir. Horizontal exchange of SCC*mec* exists in the environment as evidenced by the identification of same SCC*mec* types, such as I, III, and V, in isolates showing different PFGE patterns from multiple CoNS species. Indistinguishable methicillin-resistant *S. sciuri* in 5 meat samples from all three meat types suggest the clonal distribution of MRCoNS and the potential selective advantage of certain *S. sciuri* clones in the environment. Of particular note, many of these isolates carried SCC*mec* I. Because SCC*mec* I is relatively rare compared to other types in CoNS (Zong *et al.*, 2011) and have been identified mostly in *S. sciuri* from cattle

(Zhang *et al.*, 2009) and meat in this study, it is of great research interest to explore the association of SCC*mec* I and *S. sciuri* at the genomic level in order to understand the distribution of this SCC*mec* type in the environment. The genetic similarity among 4 *S. lentus* with SCC*mec* III from two beef samples is consistent with a previous study where indistinguishable *S. lentus* clones carrying SCC*mec* III were identified from various agricultural animals (Zhang *et al.*, 2009).

In conclusion, multiple CoNS species, including those that have been speculated as ancestral species of *mec*(A), such as *S. sciuri* and *S. fleuretti*, are prevalent in retail meat and may serve as a significant reservoir of *mec*(A) for MRSA. Clonal transmission of MRCoNS and horizontal transfer of SCC*mec* elements are common in meat. SCC*mec* I and III were associated with multiple CoNS species in different meat types, indicating broad distribution across staphylococcal species. Although SCC*mec* I, III, IV, V, and composite types were identified, many MRCoNS are still largely uncharacterized by SCC*mec* typing. Considering the extent of *mec*(A) reservoir in this group of *Staphylococcus* and that human, animals, and animal products may share common reservoir of *mec*(A) and SCC*mec*, closer investigation is needed to understand the molecular epidemiology of MRCoNS in the environment and the contribution of MRCoNS to the emergence of MRSA.

CHAPTER 4

ANTIMICROBIAL AND SYNERGISTIC POTENTIAL OF SELECTED PHYTOCHEMICALS COMBINED WITH COMMERCIALLY AVAILABLE ANTIMICROBIALS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Introduction

Infectious diseases caused by MRSA are a serious concern. This bacterium quickly develops resistance to new drugs introduced to combat its infections (Chambers and DeLeo, 2009). Although the discovery of antimicrobials had eradicated the infections that once ravaged the human kind, irrational and inappropriate use of antimicrobials provided favorable conditions for selection and spread of antimicrobial resistance resulting in development of multidrug-resistant pathogens (Levy and Marshall, 2004). There are reports on the progress of resistance to vancomycin, the last line of defense, which has led to the search for reliable methods to control MRSA infections.

Natural antimicrobials from plant are well-known as anti infectious agents. Even if plant derived antimicrobials are less potent, plant fight infections successfully as they adopt synergy to combat infections (Aiyegoro and Okoh, 2009). So, the secondary metabolites from plants are good source of combination therapy and can act as multidrug resistance modifiers (Sibanda and Okoh, 2007). Over the years, medicinal plants have been used in many different forms to treat, manage and control man's ailments, therefore the strategy to explore these ancient reservoirs for improving health care delivery would be an excellent step to troubleshoot the global problem of antimicrobial resistance (Simoes *et al.*, 2009).

Although phytochemicals especially essential oils and their purified compounds are well-known antimicrobial agents, their application is limited due to their lipophilic behavior and insolubility in water (Donsì *et al.*, 2012). Because of their limited water solubility, the undissolved essential oils applied at a concentration above the solubility limit impacts their antimicrobial efficacy. One of the strategy in dealing with such hydrophobic compounds is by dispersing them in emulsion droplets (Shah *et al.*, 2012). For emulsion systems, oil droplets can be kinetically stabilized in the continuous aqueous phase by utilizing appropriate surfactants. Majority of the studies evaluate their efficacy in broth or agar by dissolving them in ethanol or Dimethyl sulfoxide (DMSO) and there are limited efforts to address this issue (Nostro *et al.*, 2004; Gutiérrez-Larraínzar *et al.*, 2012).

Several studies have indicated the potential of phytochemicals to work synergistically with antimicrobials for which *S. aureus* has developed resistance. Catechins, Epigallocatechin gallate, tellimagrandin I and rugosin B, α-Mangostin and many other phytochemicals have been tested for their synergistic potential with commercially available antimicrobials against MRSA (Yu *et al.*, 2005; Hu *et al.*, 2001; Hu *et al.*, 2002; Takahashi *et al.*, 1995; Sakagami *et al.*, 2005). Recently, role of natural antimicrobials (carvarol, thymol, t-cinnamaldehyde) to increase effectiveness of antimicrobials against drug resistant bacteria has been explored and synergistic effect of current antimicrobials and plant derived compounds was observed (Palaniappan and Holley, 2010). However, this study fails to include MRSA, the pathogen which needs immediate attention. Exploring the natural antimicrobials as antimicrobial adjuncts for MRSA is an approach which can extend the life of successful antimicrobial drugs (Wright and Sutherland, 2007).

Although much has been learned from last decade about the antimicrobial efficacy of plant derived essential oils and natural compounds, studies on the technology for their application and evaluation of their synergistic potential with currently available antimicrobials against MRSA are still very limited. Herein, we investigated antimicrobial efficacy of antimicrobial emulsions and synergistic effect of selected phytochemicals with cefoxitin, tetracycline, erythromycin, ciprofloxacin and vancomycin against MRSA. Moreover, synergistic effect of most promising phytochemicals with commercial antimicrobials such as cefoxitin, tetracycline, erythromycin, ciprofloxacin and vancomycin against MRSA were analyzed.

Materials and methods

Bacterial Strains

Twelve strains of HA-MRSA (6) and CA-MRSA (6) recovered from human blood and retail meat were confirmed by detection of the *Staphylococcus aureus* genus and *mec*(A) gene by PCR before including them in this study (Morot-Bizot *et al.*, 2004). *S. aureus* ATCC 29213 and ATCC 43300 served as sensitive (methicillin sensitive *S. aureus*) and resistant (MRSA) control strains respectively. The MICs of cefoxitin, tetracycline, erythromycin, ciprofloxacin and vancomycin (Sigma, St. Louis, MO, USA) were determined by the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution (Wikler *et al.*, 2006).

Preparation of phytochemicals

Phytochemicals such as carvacrol, curcumin, eugenol, t-cinnamaldehyde (Sigma, St. Louis, MO, USA), garcinol (Enzo Life Sciences, USA), oregano, cinnamom, thyme, clove, lemongrass, rosemary, basil, and sage oil (LorrAnn Oils, MI) were included in this study.

These natural compounds were screened for their antimicrobial efficacy against four *S. aureus* strains including ATCC 29213, ATCC 43300, CA MRSA-2 and HA MRSA-5. Due to the insolubility of essential oils/derivatives in water, emulsions of oils in water were formulated using Tween 80 as emulsifier. Briefly, the oil and surfactant (1:0.5) were mixed and added to aqueous phase. The mixture was subjected to sonication for 10 min using an ultrasonicator (Fisher Scientific Sonic Dismembrator Model 300) at 750 W at room temperature.

Dynamic Light Scattering (DLS, in a Malvern ZetaSizer Nano ZS) was used to evaluate size and zeta potential (ζ) of the emulsions Samples (minimum of three independent batches, n = 3) were diluted 1:10 and measurements were performed at 25°C using refractive index, viscosity, and dielectric constant of DI-water (Mishra *et al.*, 2012). Zeta potential was calculated using the Smoluchowski Model. Atomic Force Microscopy (AFM, Pico SPM® LE Molecular Imaging) was used to investigate the size and morphology of the emulsions, which were formed in water. For AFM imaging, 20 μL of the emulsions dispersion were deposited on a freshly cleaved mica sheet, incubated at room temperature for 30 min to allow evaporation of the excess of water, and dried with gentle air flow. AFM images were obtained using a Multi-75 silicon AFM probe (force modulation and light tapping, purchased from Budget Sensors, 75 kHz, 3 $N.m^{-1}$) in AC tapping model (Nomani *et al.*, 2010). Mica sheet was purchased from Ted Pella Inc. Curcumin and Garcinol were dissolved in Dimethyl sulfoxide (DMSO).

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) values for these phytochemicals were determined by broth microdilution method according to National Committee for Clinical Laboratories Standards (NCCLS) guidelines. Briefly, prepared emulsions of individual essential oils (20,000 μl/L) were dissolved in MHB and serially diluted in 96-well plate. Further, fifty

microliters of the inoculums in MHB + 4% NaCl was added to the wells to obtain final concentrations of 0, 19, 39, 78, 156, 312, 625, 1,250, 2500, 5,000 and 10,000 µl/L. Similarly, Curcumin and Garcinol were dissolved in DMSO (maximum concentration 12.5%) and serially diluted in concentration from 64-0.5 µg/ml. The final concentration of bacteria in plates after addition of innoculum was 5 x 10^5 CFU/ml.

Negative controls without the respective organism and tested oils/derivatives were included to detect any cross contamination from one well to another during handling of plates. Moreover, effect of Tween 80 and DMSO alone on the growth of bacteria was examined in preliminary test and no effect was seen. Plates were incubated at 37ºC for 24 hrs and growth was monitored after 24 h using a plate reader. The MIC was determined as the lowest concentration of compound that inhibited growth with an absorbance value <0.05 at 595nm.

Synergism test

The synergism, additivity, indifference and antagonism of the antimicrobial combinations were analyzed on two multidrug resistant MRSA strains (HA and CA MRSA) and ATCC 43300. Combination testing was performed by the checkerboard method (White *et al.*, 1996). The combination antimicrobial assays were performed with selected antimicrobials (curcumin, garcinol and t-cinnamaldehyde) in combination with cefoxitin, tetracycline, erythromycin, ciprofloxacin and vancomycin. Fractional Inhibitory Concentration Index (FICI) was calculated to quantify the in vitro interaction between the drugs. The FIC index was calculated using the following formula: FICI = FICA + FICB, where FIC of compound A = MIC of compound A in combination/ MIC of compound A alone whereas FIC of compound B = MIC of compound B in combination/ MIC of compound B alone.

FIC index is the sum of these two individual FIC values and when this value is equal to or less than 0.5, the combination is termed synergistic. When FIC index value is in between 0.5-1.0 and 1.0-4.0, it indicates additivity and indifference respectively. However, if the value is more than 4 then it specifies antagonism (Mulyaningsih *et al.*, 2010).

Results

Antimicrobial efficacy of phytochemicals against MRSA

Average MIC of nano-emulsions of carvacrol, thymol, oregano, cinnamom, thyme and rosemary oil against MRSA strains were 625 µl/L followed by eugenol and lemongrass (1250 µl/L) and clove oil (5000 µl/L). Basil and sage were not effective with MICs greater than 5000 µl/L (Figure 10). Out of the fourteen phytochemicals tested, t-cinnamaldehyde emulsion (MIC: 312.5µl/L), garcinol (MIC: 2 µg/ml) and curcumin (MIC: 4-16 µg/ml) showed highest antimicrobial potential (Table 6). Therefore, they were selected for further synergism analysis with commercial antimicrobials.

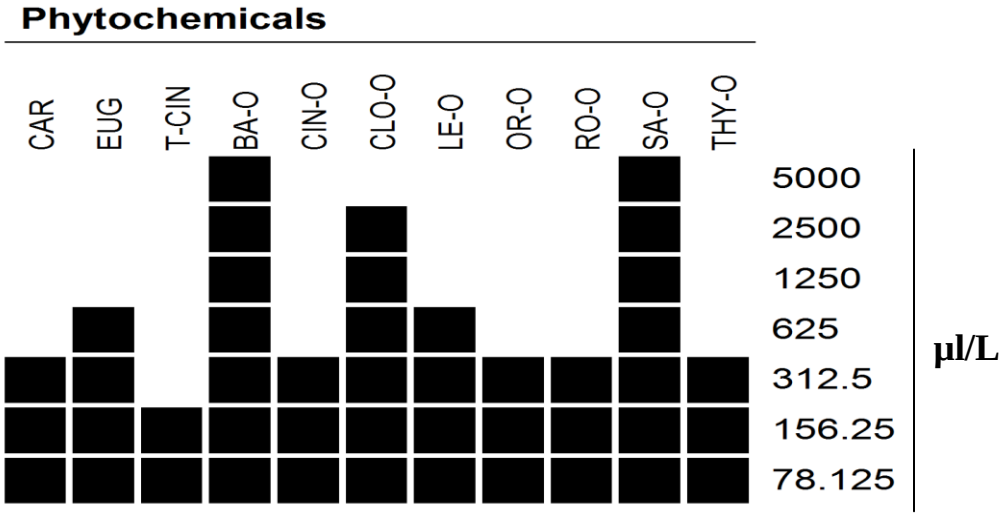


Figure 10. Minimum Inhibitory concentration (MICs) of phytochemicals nano-emulsions. Shaded black region depict bacterial growth. CAR: carvacrol; EUG: eugennol; T-CIN: t-cinnamaldehyde; BA-O: basil oil; CIN-O: cinnamom oil; CLO-O: clove oil; LE-O: lemongrass oil; OR-O: oregano oil; RO-O: rosemary oil; SA-O: sage oil; THY-O: thyme oil

Average diameter of the antimicrobial nano-emulsions was 130 nm as depicted by DLS measurements. AFM analysis showed that the droplets are evenly distributed and not agglomerated (Figure 11). Droplet size was comparable to the DLS measurement.

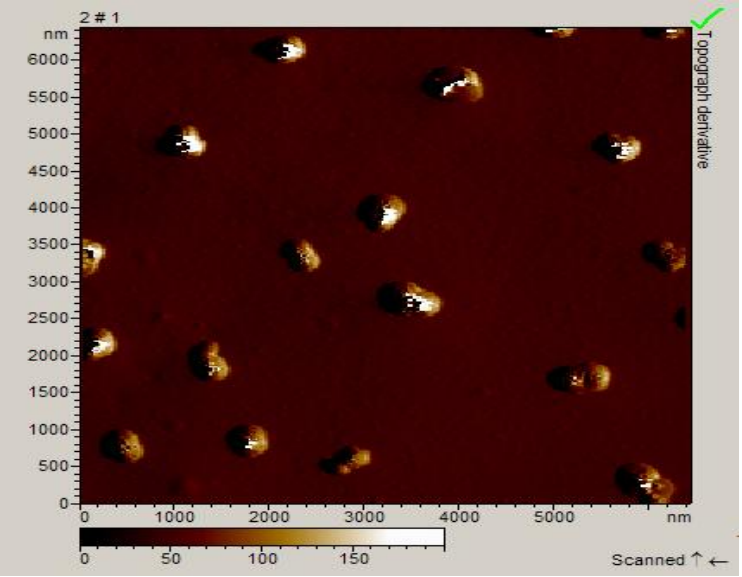


Figure 11. Representative AFM Image of oil in water antimicrobial nano-emulsions

Antimicrobial Susceptibility of HA and CA MRSA strains

Minimum inhibitory concentration for ATCC 43330 and six HA and CA MRSA isolates against cefoxitin, tetracycline, erythromycin, ciprofloxacin, vancomycin, t-cinnamaldehyde, curcumin and garcinol are shown in Table 6. All the strains were resistant to cefoxitin and MICs varied from 16- ≥64 µg/ml. Only one of the HA and CA MRSA strains (HA1 and CA5) were resistant

to tetracycline. In comparison, most of the strains were resistant to erythromycin except one strain (CAMRSA5) exhibiting sensitive phenotype. With regard to ciprofloxacin, all CA-MRSA were susceptible when compared to HA-MRSA strains where the MIC values were either 32 or ≥64. All the strains were susceptible to vancomycin and exhibited MIC values ranging from 0.5 to 2.0 µg/ml. MIC for garcinol and t-cinnamaldehyde was same 2 and 312.5 µg/ml for all strains tested. However, the MIC for curcumin varied between 4-16 µg/ml.

Table 6. Antimicrobial susceptibility of commercial antimicrobials and selected phytochemicals (t-cinnamaldehyde, garcinol and curcumin) against CA and HA-MRSA strains

MRSA strains	MIC (µg/ml)							
	CEF	TET	ERY	CIP	VAN	TCIN	GAR	CUR
ATCC43300	16	2	≥64	≤0.5	1	312.5	2	16
HAMRSA1	≥64	16	≥64	64	1	312.5	2	4
HAMRSA2	≥64	≤0.5	≥64	≥64	1	312.5	2	16
HAMRSA3	32	≤0.5	≥64	32	0.5	312.5	2	16
HAMRSA4	≥64	1	≥64	≥64	1	312.5	2	8
HAMRSA5	32	≤0.5	≥64	≥64	1	312.5	2	4
HAMRSA6	≥64	≤0.5	32	32	1	312.5	2	16
CAMRSA1	32	1	≥64	16	1	312.5	2	8
CAMRSA2	16	4	≥64	≤0.5	2	312.5	2	16
CAMRSA3	32	≤0.5	≥64	≤0.5	1	312.5	2	8
CAMRSA4	32	1	≥64	≤0.5	1	312.5	2	8
CAMRSA5	16	32	≤0.5	≤0.5	1	312.5	2	8
CAMRSA6	16	≤0.5	32	≤0.5	2	312.5	2	8
ATCC29213	4	≤0.5	0.5	≤0.5	≤0.5	312.5	2	16

Antimicrobial abbreviations: CEF: ampicilln/cefoxitin/oxacillin/pencillin; CIP: ciprofloxacin; CUR: curcumin; ERY: erythromycin; GAR: garcinol; TCIN: t-cinnamaldehyde; TET: tetracycline; VAN: vancomycin. Grey regions represent the resistance phenotype in that category according to CLSI resistance breakpoints.

Effect of phytochemicals and antimicrobial combinations

The combined effect of curcumin, garcinol and t-cinnamaldehyde with different antimicrobials (cefoxitin, tetracycline, erythromycin, ciprofloxacin and vancomycin) were examined on ATCC43300, HA4 and CA1/CA5 MRSA strain by calculation of FIC indices listed in Table 7. Curcumin and t-cinnamaldehyde lowered the MICs of antimicrobials (cefoxitin, tetracycline and erythromycin) against all 3 MRSA strains. However, no effect was observed with ciprofloxacin and vancomycin. Garcinol also exhibited null effect with all the antimicrobials tested (data not shown). Best synergistic results were observed for combination of tetracycline and curcumin (FIC=0.375) followed by t-cinnamaldehyde and cefoxitin and curcumin and erythromycin (FIC=0.5). All of the strains in these synergistic combinations showed 2 to 8 fold reduction in MIC value. Based upon FIC index, all the strains exhibited partial synergism/ additive effect for the combinations of t-cinnamaldehyde with erythromycin, and curcumin with cefoxitin. In these two cases, 2 to 4 fold reductions in MICs of commercial antimicrobials were observed. Combined antimicrobial effect of t-cinnamaldehyde and tetracycline for ATCC 43300 was found to be indifferent, however, other two CA and HA MRSA strains showed additive effect.

Table 7. Response of MRSA to combination of phytochemicals (curcumin and t-cinnamaldehyde) and commercially available antimicrobials expressed as FIC index

MRSA Strains	Antimicrobials FIC		
	Cefoxitin	Tetracycline	Erythromycin
ATCC43300			
CUR	1.0	0.50	0.50
T-CIN	0.75	1.25	1.0
HAMRSA			
CUR	1.0	0.38	0.50
T-CIN	0.50	1.0	1.0
CAMRSA			
CUR	0.75	0.38	0.50
T-CIN	0.50	0.75	1.0

Antimicrobial abbreviations: CUR: curcumin; T-CIN: t-cinnamaldehyde

Discussion

Investigators continue to search novel antimicrobial compounds due to emergence of multidrug-resistant strains of *S. aureus* worldwide. The choice of antimicrobial agents used to treat MRSA infection has been decreasing, as susceptibility of MRSA to currently available drugs is reduced by several resistance mechanisms including target-site alteration, enzyme modification, and permeability changes (Hemaiswarya *et al.*, 2008). Phytochemicals are rich source of antimicrobials and resistance modifying agents, however, their application is limited due to their insolubility in water. Therefore, in this study we screened selected phytochemicals against multidrug-resistant strains of CA and HA MRSA followed by synergism studies. Oil in water nano-emulsions of these phytochemicals demonstrated antimicrobial characteristics against

MRSA. Furthermore, synergistic combinations of some highly effective phytochemicals such as t-cinnamaldehyde and curcumin with commercially available antimicrobials were observed.

In previous studies, most of these phytochemicals are reported to demonstrate antimicrobial activity against MRSA (Chao *et al.*, 2008; Kapadia and Rao, 2011; Yang *et al.*, 2003). However, this is the first study on antimicrobial efficacy of nano-emulsions of these phytochemicals against MRSA. One of the reasons for the limited application of antimicrobial essential oil is due to their insolubility in water and most of the studies determined the antimicrobial efficacy of these phytochemicals by dissolving them in ethanol or DMSO (Gutiérrez-Larraínzar *et al.*, 2012; Palaniappan and Holley, 2010).

Comparisons with the previous reports on anti-MRSA activity of these phytochemicals are difficult because of different solvents and experimental conditions utlized. However, in most of the reports t-cinnamaldehyde exhibited maximum efficacy followed by carvacrol, thymol and eugenol. All of them are phenols and are known for their highest efficacy in comparison to other essential oil components classified as aldehydes, ketones, alcohols and hydrocarbons (Kalemba and Kunicka, 2003). Therefore, highest efficacy of t-cinnamaldehyde against MRSA in our study was not surprising as it exerts antimicrobial effect by several mechanisms, including disruption of carbohydrate, aminoacids and lipid metabolism (Simoes *et al.*, 2009). Our MIC results for carvacrol and thymol were consistent with previous report in which these compounds exhibited MIC of 0.6 and 1.25 µl/ml respectively (de Oliveira *et al.*, 2010).

Lemongrass, rosemary, bay, basil and sage oil have been shown to exhibit antimicrobial potential against MRSA in previous study (Chao *et al.*, 2008). The efficacy was tested utilizing disc-diffusion test and reported diameter of inhibition was highest for lemongrass oil followed by rosemary, basil, bay and sage. Almost similar findings were observed in our study, however, the

antimicrobial efficacy of bay oil was higher than basil. This difference may be attributed to variability in sources of oils carrying these antimicrobial components. Lemongrass oil has been reported to exhibit antimicrobial efficacy by broth dilution method and the MIC value was close (600µl/L) to our findings, although, this study didn't follow the CLSI guidelines and experiments were performed in BHI broth. Similarly, rosemary oil was found to be highly effective against *S. aureus* and showed MIC value between 300-1000µl/L (Jiang *et al.*, 2011). Although, antimicrobial efficacy of bay oil against MRSA has been demonstrated by disc diffusion method previously (Sharma *et al.*; Chao *et al.*, 2008), there is no report on the MIC for this oil against MRSA in literature till date.

Strong antimicrobial efficacy of Curcumin (polyphenol) and garcinol (polyisopreneylated benzophenone derivative) was not unexpected as recent studies have demonstrated their antimicrobial potential (Acuna *et al.*, 2009; Kim *et al.*, 2005). Phenols and phenolic acids exerts antimicrobial effect either through disruption of energy production due to enzyme inhibition by oxidized products or through non-specific interactions with proteins (Simoes *et al.*, 2009). In comparison, benzophenone target the bacterial membrane and disrupts membrane potential (Vooturi *et al.*, 2011). Our MIC results for garcinol were consistent with the finding of Negi et al. where 1.5 µg/ml of garcinol inhibited *S. aureus* by agar dilution method (Negi and Jayaprakasha, 2004). However, other study with garcinol and MRSA reported higher MIC of 16 µg/ml (Rukachaisirikul *et al.*, 2005). MIC for curcumin was lower than the previous study on curcumin and MRSA (Mun *et al.*, 2013) which may be attributed to difference in extraction method for curcumin.

We decided to perform synergism studies with t-cinnamaldehyde, curcumin and garcinol because of their low MICs and no previous reports on their synergism potential. Gracinol

showed null effect with all antimicrobials tested, although, the MIC of antimicrobials was decreased to 2µg/ml (MIC of garcinol) with addition of garcinol. Therefore, we assume that bacterial growth was inhibited primarily because of garcinol.

With respect to curcumin, it showed antimicrobial and synergistic/additive effect with tetracycline and erythromycin when used alone and in combination in all tested strains. Previous study has demonstrated synergistic effects of curcumin against MRSA, however, they tested curcumin against ampicillin, oxacillin, ciprofloxacin and norfloxacin (Mun *et al.*, 2013). Curcumin is a polyphenol and potential of phenols such as epigallocatechin, α-mangostin and corilagin as a resistance modifying agents has been demonstrated in previous reports (Yu *et al.*, 2005; Hu *et al.*, 2001; Hu *et al.*, 2002; Shimizu *et al.*, 2001). As far as we know, this is the first report investigating synergistic potential of t-cinnamaldehyde and garcinol with standard antimicrobials against MRSA. t-cinnamaldehyde have been tested against *blaZ* positive *S. aureus* in previous study and synergism was observed with ampicillin, penicillin and bacitracin (Palaniappan and Holley, 2010).

In conclusion, antimicrobial efficacy demonstrated by emulsions of these phytochemicals indicates potential of their use in topical MRSA ointments. Our results showed that curcumin and t-cinnamaldehyde were able to decrease the MIC of commercial antimicrobials such as cefoxitin, tetracycline and erythromycin in both HA and CA-MRSA strains. Both of them were either synergistic or showed additive effect with standard antimicrobials indicating that they offer huge potential as alternative phytotherapy against MRSA. Further studies are needed to evaluate the mechanism of action and the efficacy of these results in vivo models.

SUMMARY AND FUTURE DIRECTIONS

Staphylococci are ubiquitous in nature, which gives them opportunities to share common reservoir in food, environment, and human clinical settings. Antimicrobial resistance in these pathogenic commensals is a serious public health concern worldwide. Methicillin-resistant *S. aureus* (MRSA) is an example of superbugs which has emerged worldwide as a signature of antimicrobial resistance problem in Staphylococci. Food production environment is an important reservoir of multidrug-resistant *Staphylococcus* spp. and their molecular composition can differ by meat and animal origin. Importantly, these pathogens may be transmitted from the agriculture environment to humans. The research described in this dissertation focused on understanding the molecular epidemiology, and antimicrobial resistance reservoir of this pathogen in retail meat and food animals. Furthermore, potential application of phytochemicals as antimicrobials and antimicrobial adjuvants to control MRSA infections was explored.

In chapter 1, a total of 87 CoNS recovered from food animals were characterized by antimicrobial susceptibility testing, resistance gene identification and conjugation. Of the seven species studied, *Staphylococcus lentus*, *Staphylococcus sciuri*, *Staphylococcus xylosus* and *Staphylococcus haemolyticus* accounted for over 96% of the isolates. In addition to β-lactam resistance (100%), high percentages of CoNS were resistant to tetracycline (67·8%), erythromycin (36·7%), clindamycin (27·5%) and quinopristin/dalfopristin (14·9%). Importantly, 47 (54%) isolates were resistant to at least three antimicrobial classes, including six CoNS resistant to six antimicrobial classes. The common genes for the above-mentioned resistance phenotypes were *mec*(A), *tet*(M), *erm*(A) and *vga*$(A)_{LC}$, which were identified from 68·7%, 61%, 56·2% and 69·2% of the isolates, respectively. *tet*(M) was conjugatively transferable from

10 tetracycline-resistant CoNS to a *Enterococcus* strain, underlining the potential of antimicrobial resistance transfer from *Staphylococcus* to the commensal bacteria in human.

Our data indicate that CoNS in agriculture animals are an important reservoir of multidrug resistance in addition to the resistance to β-lactam antimicrobials and underline the importance of surveillance of multi-drug resistant CoNS in food production environment. Since our CoNS strains were all resistant to at least one β-lactam antimicrobial, further research is needed as to whether methicillin resistance predisposes CoNS to become multidrug-resistant as compared to general CoNS, including the potential linkage, if any, between β-lactam resistance and other resistance phenotypes. Species variation exists in the prevalence of multidrug resistance in Staphylococci. Certain Staphylococcal species, such as S. *haemolyticus*, may have stronger potential to become multidrug resistant and thus require closer research and public health attention.

In chapter 2, we isolated and characterized *S. aureus* including MRSA and MSSA from retail meat. *S. aureus* was recovered from 65 out of 289 meat samples including 6 samples carrying MRSA. Nine MRSA isolates recovered were resistant to atleast one non β-lactam antimicrobial, including one isolate which was multidrug-resistant indicating that non β-lactam resistance is also prevalent in MRSA of food origin. All MRSA isolates were human clone, suggesting a possible human contamination. They all exhibited similar molecular profiles (SCC*mec* IV, ST8, *pvl* positive and *agr*I) by various subtyping methods, except for *spa* typing, which identified 2 distinct *spa* types, t008 and t2031. Recovery of t2031, a *spa* variant of USA300, from a chicken product raises questions on its emergence, antimicrobial resistance and virulence potential. Further, prevalence of common MRSA ST types ST8, ST5, ST9 and ST72 recovered from meat

in MSSA, with three strains of MSSA ST8 exhibiting 80% similarity to USA 300 strains, suggests that MSSA in meat have the potential to become MRSA once acquiring *mec*(A).

In chapter 3, we investigated the role of MRCoNS in the emergence of MRSA by characterizing the MRCoNS recovered from the same niche as MRSA. Multiple CoNS species, including those that have been speculated as ancestral species of *mecA*, such as *S. sciuri* and *S. fleuretti*, are prevalent in retail meat and may serve as a significant reservoir of *mecA* for MRSA. Clonal transmission of MRCoNS and horizontal transfer of SCC*mec* elements are common in meat. SCC*mec* I and III were associated with multiple CoNS species in different meat types, indicating broad distribution of common SCC*mec* types across staphylococcal species. Although SCC*mec* I, III, IV, V, and composite types were identified, many MRCoNS were still largely uncharacterized by SCC*mec* typing. Considering the extent of *mec*(A) reservoir in MRCoNS and that human, animals, and animal products may share common reservoir of *mec*(A) and SCC*mec*, closer investigation is needed to understand the molecular epidemiology of MRCoNS in the environment and the contribution of MRCoNS to the emergence of MRSA. While the role of retail meat as a vehicle for *S. aureus* and MRSA infections is still undetermined, the presence of potentially virulent strains of MRSA such as *pvl* positive t008 suggest that this mode of transmission needs further investigation cannot be ignored.

In chapter 4, antimicrobial efficacy and synergistic potential of selected natural phytochemicals were evaluated against HA and CA MRSA strains. Most of the phytochemicals, tested in the form of nano-emulsions, demonstrated anti-MRSA activity. However, t-cinnamaldehyde emulsion (MIC: 312.5µl/L), garcinol (MIC: 2 µg/ml), and curcumin (MIC: 4-16 µg/ml) showed highest antimicrobial potential. Therefore, they were selected for further synergism analysis with commercial antimicrobials such as cefoxitin, tetracycline, erythromycin,

ciprofloxacin, and vancomycin. Curcumin and t-cinnamaldehyde showed either synergistic or additive interaction with cefoxitin, tetracycline, erythromycin, whereas, no effect was observed with garcinol. Antimicrobial nano-emulsions of phytochemicals and their combinations with commercial antimicrobials offer alternatives to control MRSA infections. Further studies on synergistic mechanism of these phytochemicals with commercial antimicrobials will provide an insight to control this multidrug-resistant pathogen.

CONCLUSIONS

Food production environment including food animals and retail meat are important reservoir of antimicrobial resistant *Staphylocccus* spp. The molecular epidemiology of this pathogen in food chain, however, is still mostly unknown. Antimicrobial resistant *Staphylococcus* spp. may be transmitted from the agriculture environment to humans leading to public health implications. Here, I have characterized antimicrobial resistant *Staphylocccus* spp. to understand the molecular epidemiology, and antimicrobial resistance reservoir of this pathogen in retail meat and food animals. Furthermore, potential application of phytochemicals as antimicrobials and antimicrobial adjuvants to control MRSA infections was explored.

Food animals and retail meat provide a diverse reservoir of antimicrobial-resistant *Staphylococcus* spp. Multidrug resistance and resistant determinants are common in CoNS in food animals. Further, some of the resistant determinants may transfer to other commensals or human pathogens. The presence of MRSA and MRCoNS in meat may pose potential threat of infection to individuals who handle the meat. The molecular composition of MRCoNS and MSSA strains clearly represent genetic background favorable for the emergence of MRSA. Recovery of these superbugs from food animals and retail meat is a public health concern. Antimicrobial nano-emulsions of phytochemicals and their combinations with commercial antimicrobials offer alternatives to control MRSA infections.

The data improve our understanding on the extent to which antimicrobial resistant *staphylococcus* spp. contribute to the dissemination of antimicrobial resistance in the food production environment.

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ABSTRACT

CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT *STAPHYLOCOCCUS* SPP. IN FOOD ANIMALS AND RETAIL MEAT

by

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Antimicrobial resistance in *Staphylococcus* spp. is a worldwide epidemic concern in hospital and community settings. Food animals and retail meat are important reservoirs of these pathogens that can pose potential threat to humans. In this dissertation, we aimed to investigate the molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant CoNS (MRCoNS) in food animals and retail meat to provide insight into the role of agricultural environment in transmitting bacteria of human clinical significance. Furthermore, the potential application of phytochemicals as antimicrobials and antimicrobial adjuvants to control MRSA infections was explored.

CoNS recovered from food animals were characterized by antimicrobial susceptibility testing, resistance gene identification and conjugation. *Staphylococcus lentus, Staphylococcus sciuri, Staphylococcus xylosus* and *Staphylococcus haemolyticus* accounted for over 96% of the isolates. Resistance to macrolides, tetracyclines, and Q/D, in addition to β-lactam resistance was observed with 54% isolates classified as multi-drug resistant. *tet*(M) was conjugatively transferable from 10 tetracycline-resistant CoNS to other commensals like *Enterococcus faecalis* by conjugation.

S. aureus was recovered from 65 of 289 meat samples, including 6 samples carrying MRSA. All MRSA isolates were USA 300, the most common community-associated MRSA clone. They exhibited similar molecular profiles (SCC*mec* IV, ST8, *pvl* positive and *agr*I) by various sub typing methods, except for *spa* typing, which identified 2 distinct *spa* types, t008 and t2031. Multiple CoNS species, including those that have been speculated as ancestral species of *mec*(A), such as *S. sciuri* and *S. fleuretti*, were prevalent in retail meat. From our SCC*mec* typing experiments, we identified SCC*mec* types IV and V in MRCoNS which has been previously found in MRSA from meat.

Most of the phytochemical emulsions demonstrated antimicrobial activity against MRSA. Curcumin, t-cinnamaldehyde and garcinol (MIC: 4-16µg/ml, 312.5µl/L and 2µg/ml) were highly effective in inhibition, whereas, t-cinnamaldehyde and curcumin combinations showed synergistic or additive effect with commercial antimicrobials (cefoxitin, tetracycline and erythromycin) against MRSA.

In conclusion, food animals and retail meat provide a diverse reservoir of antimicrobial-resistant *Staphylococcus* spp. Multidrug resistance is common in CoNS in animals. The presence of MRSA and MRCoNS in meat may pose potential threat of infection to individuals who handle the meat. The molecular composition of MRCoNS and MSSA strains clearly represent genetic background favorable for the emergence of MRSA. Antimicrobial nano-emulsions of phytochemicals and their combinations with commercial antimicrobials offer alternatives to control MRSA infections.

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